Molecular genetics and physiological characterization of postharvest quality of potted roses (*Rosa hybrida* L.)

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von
Mohammad Mahmoud Mousa Al-Salem, M.Sc. (Jordanien)
geboren am 30.08.1975 in Rhaba, Jordanein

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Referentin: [Prof. Dr. Margrethe Serek]

Korreferent: [Prof. Dr. Edgar Maiss]

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ZUSAMMENFASSUNG

Miniatur-Topfrosen gehören zu den beliebtesten und attraktivsten Topfpflanzen, deren Popularität in den letzten Jahren stark zugenommen hat. Sie sind in den unterschiedlichsten Blütenfarben, -formen, und -größen sowie Wuchsformen erhältlich. Floristen und Rosenzüchter zeigen ein großes Interesse an Miniatur-Topfrosen mit verbesserten Qualitätsmerkmalen. Vor allem haben Haltbarkeit und Nachernteverhalten der Miniaturrose in der Zierpflanzenproduktion eine große ökonomische Bedeutung. Dabei ist das Phytohormon Ethylen einer von vielen Faktoren, der die Qualität, das Aussehen und die Langlebigkeit der Miniaturrosen beeinflusst.

In der vorliegenden Studie wurde die Expression von Ethylen-assoziierten Genen aus der Biosynthese, der Perzeption, der Signaltransduktion und Transkription in verschiedenen Stadien der Blütenentwicklung in den beiden Sorten "Vanille" und "Lavender", die jeweils eine niedrige und hohe Ethylenempfindlichkeit aufweisen, und ihrer F1-Generation untersucht. Mit Hilfe der Zwei-Schritt Reverse Transkriptase-Polymerase-Kettenreaktion (RT-PCR), eines nicht-radioaktiven Northern Blot-Verfahrens und des Reverse Northern Blots wurden Expressionsprofile von ausgewählten Gene untersucht. Nur die Gene, die zwischen beiden Sorten signifikante Unterschiede zeigten, wurden anschließend analysiert, um Einzelnukleotid-Polymorphismen (SNP) Marker unter Verwendung von CAPS (*cleaved amplified polymorphic sequence*, mit Restriktionsendonukleasen gespaltene amplifizierte polymorphe Sequenzen) zu finden. Gene für die Ethylenrezeptoren RhETR1, RhETR2 und RhETR3, Gene für die Rezeptor-assoziierten Signalproteine RhCTR1 und RhCTR2, Gene für die Transkriptionsfaktoren RhEIN3 und RhEIL sowie Gene für die ACC-Synthasen RhACS1 und RhACS2 wiesen jeweils ein Expressionsmuster auf, dass zwischen den getesteten Pflanzen und Geweben varriert, nicht aber mit der Ethylen-empfindlichkeit der Pflanzen korreliert. RhETR1, RhETR2, RhETR3 und RhEIN3 wurden

beispielsweise in "Vanille" stärker exprimiert als in "Lavender", jedoch nicht in allen untersuchten Geweben. Im Allgemeinen zeigt sich aber, dass die Nachkommenschaften mit verringerter Ethylenempfindlichkeit aber auch eine geringere Expression der Rezeptorgene im Vergleich zu den Nachkommen mit erhöhter Empfindlichkeit haben. Für die anderen 4 untersuchten Gene – RhETR4, RhACS3, RhACS4 und RhACS5 – konnte keine Expression nachgewiesen werden. Daher wurden die Gene RhETR3 und RhEIN3 kloniert, sequenziert und analysiert, um gezielt SNP-Marker zu finden. Auf diese Weise konnten drei SNP-Marker in partiellen DNA-Sequenzen der RhETR3-Allele und zwei SNP-Marker in RhEIN3-Allelen identifiziert und validiert werden. Obwohl die CAPS-Methode erfolgreich war und mehrere SNPs-Marker nachgewiesen werden konnten, ergab sich keine Beziehung zwischen den detektierten SNPs und einer Ethylenempfindlichkeit. Es wird daher vermutet, dass die untersuchten Gene nicht direkt mit der Empfindlichkeit der Pflanze gegenüber Ethylen in Zusammenhang stehen und andere Gene für die Variation der Ethylenempfindlichkeit verantwortlich sein könnten.

Daraus kann gefolgert werden, dass die eigentliche Transkriptionsaktivität der getesteten Gene für eine Bestimmung der Ethylenempfindlichkeit in Miniaturrosen nicht entscheidend ist. Es scheint daher wahrscheinlich, dass die Transkription weiterer Gene aus der Ethylen-Signaltransduktion, der posttranskriptionellen bzw. posttranslationalen Regulation oder auch eine Überschneidung mit anderen Signaltransduktionswegen die eigentliche Empfindlichkeit von Miniaturrosen gegenüber Ethylen maßgeblich beeinflussen.

Schlüsselwörter: Genexpression, Ethylenempfindlichkeit, Nachernte, Haltbarkeit, molekulare Marker, CAPS.

ABSTRACT

Miniature potted roses are among the most charming and delightful of potted flowers, they have become popular in recent years, and they are available in a wide range of colors, sizes, bloom styles, and growth habits. One main interest of floriculturist and rose breeders is to improve the quality of the miniature-potted roses; for this reason, postharvest life of potted miniature roses is of great economic importance in ornamental flower industry. Ethylene is one of many factors that affect the quality, appearance and longevity of miniature roses. In this study, the expression of ethylene biosynthetic enzymes genes, ethylene receptor genes and ethylene signal transduction & transcription genes at different stages of flower development in the two cultivars 'Vanilla' and 'Lavender', which show low and high ethylene-sensitivity, respectively, and their F1 offspring were investigated. Two steps reverse transcriptase polymerase chain reaction (RT-PCR), non-radioactive northern blot hybridization and reverse northern dot blot were used to investigate the expression patterns of selected genes. The genes that showed remarkable differences between the two cultivars subsequently analyzed to find single nucleotide polymorphism (SNP) Markers by using cleaved amplified polymorphic sequences (CAPS) method. The genes for the ethylene receptors RhETR1, RhETR2 and RhETR3, the genes for the receptor-associated signaling proteins RhCTR1 and RhCTR2, the genes for the transcription factors RhEIN3 and RhEIL, and the genes for the ACC synthases RhACS1 and RhACS2 each had an expression pattern that varied between the tested plants and tissues, but could not be correlated with the ethylene sensitivity of the plants. RhETR1, RhETR2, RhETR3 and RhEIN3 were, e.g., expressed more in 'Vanilla' than in 'Lavender' in most, but not all, of the investigated tissues, but were in general not expressed more in progeny with low sensitivity than in progeny with high sensitivity. No expression was detected for the 4 other genes that were investigated, i.e. genes for RhETR4, RhACS3, RhACS4 and

RhACS5. Therefore, RhETR3 and RhEIN3 were cloned, sequenced and analyzed to find SNP Markers. Three SNP markers in partial DNA sequence of the RhETR3 alleles and two SNPs marker in RhEIN3 alleles were identified and validated. Although CAPS method was successful and several SNPs marker was detected, there were no relationship between the SNPs and ethylene sensitivity. This might be explained as the investigated genes may not related directly to the ethylene sensitivity and other genes might be responsible for the variation for the ethylene sensitivity. It is concluded that the precise transcriptional activities of the tested genes do not appear to be crucial in determining the ethylene sensitivity of miniature roses. It therefore appears likely that transcription of other genes involved in ethylene signal transduction, posttranscriptional or posttranslational control, or crosstalk with other signal transduction pathways may be important for the degree of ethylene sensitivity of miniature roses.

Keywords: gene expression, ethylene sensitivity, postharvest, display life, molecular marker, CAPS

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DEDICATION

To my Family…

…with love

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When I traveled to complete my graduate studies, I could not imagine that the road was very long and winding. I could have never completed this study without the help of so many wonderful people. Words will not express the real efforts and can never thank them enough. Special thanks go to my supervisor Prof. Dr. Margrethe Serek for her encouragement, valuable comments, inimitable sponsorship, and suggestions that lead to advance this project. I am very indebted to you Prof. Serek. I would also like to direct gratitude to Dr. Heiko Mibus for his advices in the Lab.

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LIST OF ABBREVIATIONS

°C	degree Celsius
1-MCP	1-Methylcyclopropene
ABA	Abscisic acid
ACC	1-amino cyclopropane-1-carboxylic acid
ACS	1-aminocyclopropane-1-carboxylate synthase
AHP	ARABIDOPSIS HIS PHOSPHOTRANSFER
bp	base pair
BSA	bovine serum albumin
CAPS	cleaved amplified polymorphic sequence
Cb5	cytochrome b5
cDNA	complementary deoxyribonucleid acid
CO2	Carbondioxide
CTR	Constitutive triple response
CTR1	Constitutive Triple Response 1
cv.	Cultivar
DACP	Diazocyclopentadine
dATP	Deoxyadenosinetriphosphate
dCTP	Deoxycytosinetriphosphate
DDRT-PCR	differential-display RT-PCR
dGTP	Deoxyguanosinetriphosphate
DNA	Deoxyribose nucleic acid
DNAase	Deoxyribonuclease
dNTP	Deoxyribonucleotide tri-phosphate
dTTP	Deoxythymidinetriphosphate
E. coli	*Escherichia coli*
EBF1	ETHYLENE INSENSITIVE3 BINDING F-BOX1
EDTA	Ethylene diamine tetra acetate
EIL	EIN3-like
EIN	Ethylene insensitive
EIN2	Ethylene Insensitive 2
EIN3	Ethylene Insensitive 3
EIN4	Ethylene Insensitive 4
ER	Endoplasmic Reticulum
ERF	Ethylene response factor
ERS	Ethylene response sensor
ERS1	Ethylene Response Sensor 1
ERS2	Ethylene Response Sensor 2
EST	expressed sequence tag
EtBr	ethidium bromide
ETP1	ETHYLENE INSENSITIVE2 TARGETING PROTEIN1
etr	ethylene resistant mutant
ETR1	Ethylene Response gene 1
ETR2	Ethylene Response 2
GC	Gas chromatograph
h	Hour
HK	His kinase

IAA	indoleacetic acid
IPTG	Isopropyl-β-D-thiogalactopyranosid
kb	kilo base
KD	kinase domain
LAC	Laccase
LB	medium Luria-Bertani medium
LG	linkage group
M	Molar
MAP	mitogen-activated protein
min	minute(s)
mRNA	messenger RNA
NCBI	National center for biotechnology information
NCED	9-cis-epoxycarotenoid dioxygenase
Nr	Never ripe (LeETR3)
ORF	open reading frame
PAL	Phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PDS	phytoene desaturase
PFGE	Pulsed field gel electrophoresis
QRT-PCR	quantitative reverse transcriptase real-time PCR
QTL	quantitative trait loci
RACE	rapid amplification of cDNA ends
RAN1	RESPONSIVE TO ANTAGONIST1
RFLP	restriction fragment length polymorphism
RH	Relative humidity
RNA	Ribose nucleic acid
RNase	Ribonuclease
RTE1	REVERSION-TO-ETHYLENE SENSITIVITY1
RT-PCR	Reverse transcriptase polymerase chain reaction
s	second(s)
SAG	senescence-associated gene
SAM	S-adenosyl methionine
SCAR	sequence characterized amplified region
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
STS	Silver thiosulfate
TAE	buffer Tris-Acetate-EDTA buffer
Taq	Thermus aquaticus
Tris	tris-(hydroxymethyl) aminomethane
X-Gal	5-bromo-4chloro-3-indolyl-ß -D-galactopyranoside
XRN4	EXORIBONUCLEASE4

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CHAPTER 1

INTRODUCTION

1 INTRODUCTION

Flowering potted plants have been increasingly distributed in markets along with the increase of flowers production in recent years. Miniature potted roses are among the most charming and delightful of potted flowers, they have become popular in recent years, and they are available in a wide range of colors, sizes, bloom styles, and growth habits. In addition, their small size makes them useful to plant in containers, pots, and small gardens. The genus Rosa is very large and includes about 200 species and more than 18,000 cultivars (Gudin, 2000). More than 100 million pots of miniature roses are produced annually in the world; Denmark is the largest producer with nearly 50 % of the world's production (Pemberton et al. 2003). The number of miniature rose plants sold in 2015 at Flora Holland, the world largest flower auction, reached 47 million, generating revenues of EUR 57 million (Flora Holland, 2016).

Postharvest quality is one of the highly desirable trait that rose breeders are selecting for (Pemberton et. al. 2003). Quality and longevity have been primary issues in research regarding increased flower sales and improved flower products for consumers. One main interest of floriculturist and rose breeders is to improve the quality of the miniature-potted roses; for this reason, postharvest life of potted miniature roses is of great economic importance in ornamental flower industry. Many factors affect the quality, appearance and longevity of potted miniature roses, such as cultivars, temperature, light, food and water supply, disease and ethylene. Many physiological and developmental processes such as germination, flower and leaf senescence, fruit ripening, leaf abscission, root nodulation, programmed cell death, and responsiveness to stress and pathogen attack are regulated by the gaseous plant hormone ethylene (Abeles et al, 1992, Alonso and Ecker, 2001, Bleecker and Kende, 2000). Ethylene is one of the most important factors affecting the quality, appearance and longevity of many ornamentals (Serek et al. 2006, Ferrante et al. 2015). In miniature roses, ethylene can cause unwanted effects such as premature and

accelerated wilting and abscission of leaves, floral buds, petals and flowers, as well as leaf yellowing or discoloration (Serek 1993, Andersen et al. 2004). Due to harmful effect of ethylene on potted plants, ornamental flower industry has been working on ways to prevent damage caused by ethylene. Generally, these include preventing ethylene pollution, removing ethylene from the atmosphere, and inhibiting the production of ethylene and its action. The display life of some commercially grown miniature potted rose cultivars was compared by Müller et al. (1998), and it was found that it varied considerably, at least partly due to differences in endogenous ethylene production and sensitivity to exogenous ethylene. The degree of sensitivity to ethylene varies with plant species, variety, ethylene concentration, and temperature during exposure and duration of exposure (Woltering, 1987). Cultivars, production season, initial plant quality, and duration of simulated transport have a certain disposition towards the postharvest longevity of miniature roses (Borch et. al.1996).

In higher plants, the mechanism of ethylene biosynthesis has been well-investigated (Yang and Hoffman, 1984, Xu and Zhang 2015). Ethylene signal transduction has also been investigated extensively, resulting in a model encompassing the following well-established aspects (Figure 1), (Merchante et al. 2013, Cho and Yoo 2015, Gallie 2015, Ju and Chang, 2015, Shakeel et al. 2015). One distinguishing characteristics of the ethylene response system is that the receptors are considered to be constitutively active. In the absence of ethylene, the ethylene receptors family, located in the ER membrane, activate the kinase activity of negative regulator CTR1 (and its homologs) which is associated with the receptors and, when activated, phosphorylates and inhibits EIN2 (a membrane protein also located in the ER membrane) and leads to repression of the ethylene response pathway. (Figure 1). In the presence of ethylene, CTR1 is "switched off", and EIN2 becomes dephosphorylated, leading to release of its C-terminal domain (Ju et al. 2012). This domain translocates to the nucleus, where it blocks degradation of the transcription factors

EIN3/EIL1 and thus activates the transcriptional response to ethylene. Other, less well-characterized proteins, also are involved in the signal transduction pathway (Cho and Yoo 2015). In summary to this model, The RAN1 protein is essential for assemble the Cu cofactor with the receptor for C2H4 binding. In the absence of ethylene, the receptor would activate the downstream kinase CTR1 that in turn inhibits the response/gene expression, while in the presence of ethylene, the receptor is "inhibited" so is the CTR1 kinase, the response is now "on". The kinase cascade may serve as a negative regulator of the gene expression. Depending on this model we can expect the following effects of changes in receptor and CTR1 protein levels, an increased level of ethylene-receptor proteins would (if there is enough CTR1 protein to bind all the receptor molecules, so that more receptor results in more receptor-CTR1 complexes) mean that more ethylene is required to inactivate all the receptor-CTR1 complexes so that the ethylene-response is started. Thus, the plants are less ethylene-sensitive when there is more receptor protein. In the hypothetical case of no receptor protein, CTR1 would be permanently switched off and the ethylene pathway would be permanently switched on - even when ethylene is absent. This could be said to represent ultimate sensitivity. In the hypothetical case of very high levels of receptor protein, CTR1 would be permanently switched ON (unless there are very high levels of ethylene) and the ethylene pathway would be permanently switched OFF - even when ethylene is present in normal amounts. This could be said to represent ultimate lack of sensitivity. Increased levels of CTR1 proteins would (if there is enough receptor protein to bind all the CTR1 molecules, so that more CTR1 results in more receptor-CTR1 complexes) mean that more ethylene is required to inactivate all the receptor-CTR1 complexes, so that the ethylene-response is started. Thus, the plants are less ethylene-sensitive when there is more CTR1.

There are several ethylene receptors (5 in Arabidopsis) with partly overlapping function (Shakeel et al. 2013, Gallie 2015). From this model, it may be expected that increased amounts of receptor

and CTR1 proteins would lead to less ethylene-sensitive plants, whereas increased amounts of EIN2 and/or EIN3/EIL1 proteins would lead to more ethylene-sensitive plants.

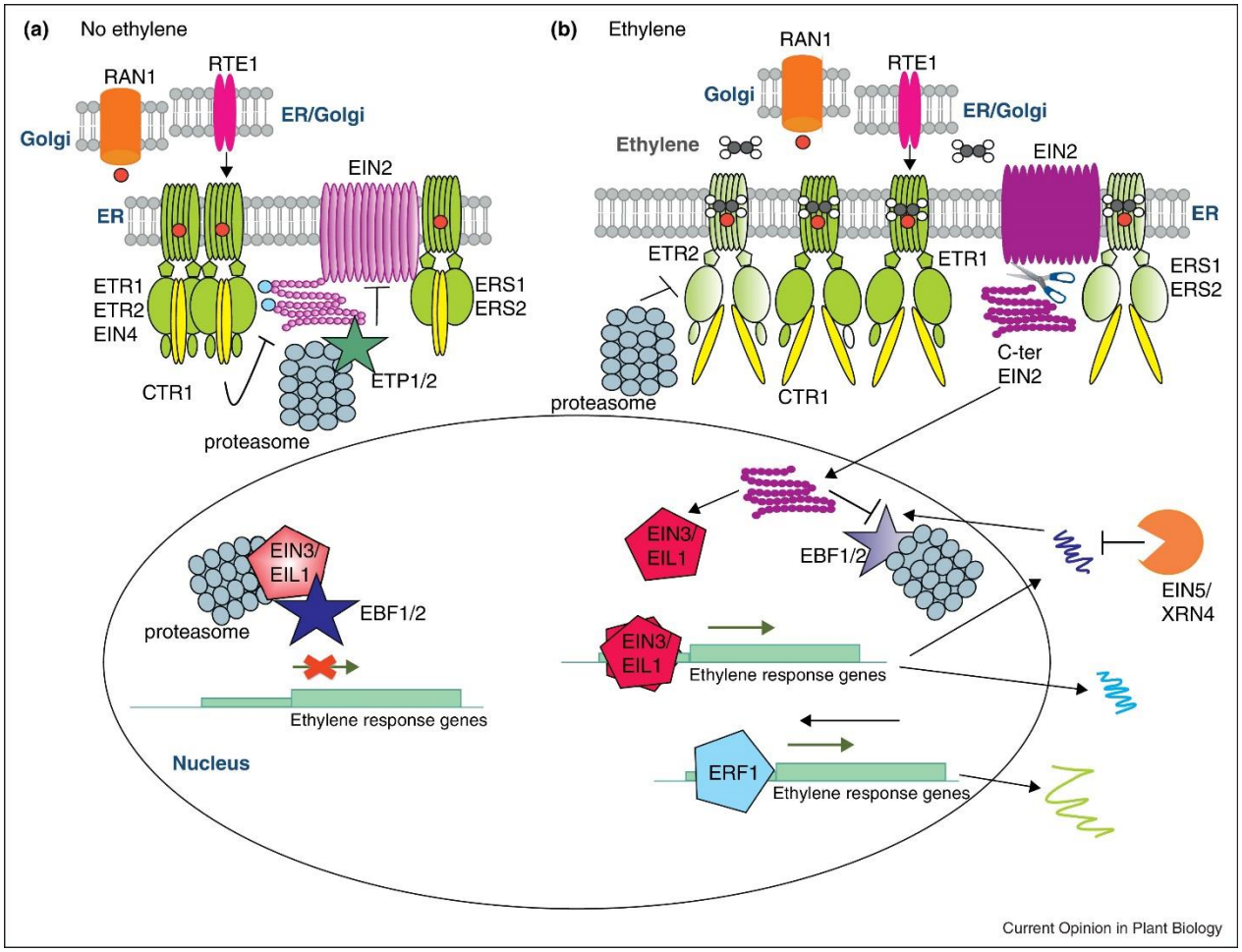


Figure 1.
Schematic model of the ethylene signaling pathway. In the absence (a) or presence (b) of ethylene perception. (Merchante et al. 2013).

Müller et. al. (1998) compared the display life of some commercial potted rose cultivars and found that they varied in their postharvest life, at least partly due to the differences in endogenous ethylene production, and in sensitivity to exogenous ethylene. It is of great importance to know the reasons for such differences in postharvest life of miniatures roses, therefore some researches were conducted to examine the reasons for differences in flower longevity in miniature rose cultivars by investigating the expression of transcripts for ethylene biosynthetic enzymes genes, ethylene

receptor genes and ethylene signal transduction & transcription genes (Müller et. al. 2000a, 2000b, 2001a, 2001b, 2002, 2003). Understanding the expression of those genes provides more information about the postharvest life of miniature potted roses and thus to certain extent explain the sensitivity of different miniature rose cultivars to ethylene. Some cultivars are highly susceptible to ethylene; some other cultivars are relatively less sensitive. In this study, two cultivars of potted miniature rose were chosen; 'Vanilla' which appears to be low sensitive to ethylene and 'Lavender' which is high sensitive to ethylene. As reported by Müllar et. al. (1998) 'Vanilla' or similar (low sensitive to ethylene) cultivars may be certainly useful genetic resources for improving the postharvest life of miniature potted rose by mean of conventional breeding methods or molecular genetic techniques.

In conventional plant breeding program, the desirable parental genotypes crossed together and then the marketable plants with new or desirable characteristics will be selected. In recent years, plant breeding is much advanced in terms of hybridization and selection procedures, but several problems are still unsolved such as introducing only the gene(s) of interest into their cultivated plants because conventional breeding methods rely on the transfer of whole genome. This means that along with gene of interest, undesirable characters will also be co-inherited (Farooq and Azam, 2002).

With the advent of molecular biology, techniques are being developed that greatly be useful for plant breeding programs. In many cases those techniques reduce the amount of time and money required for the development of new cultivars. One of the most extensive uses of these molecular markers has been the development of detailed genetic and physical chromosome maps in a variety of organisms, including, among the animal systems, humans and, among the plant systems (Gupta et. al. 1999). Another important application of molecular markers in plant systems involves improvement in the efficiency of conventional plant breeding by carrying out indirect selection through molecular markers linked to the traits of interest because these markers are not influenced

by the environment and can be scored at all stages of plant growth In addition to these two major applications, DNA markers can also be used in plant systems for germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization, phylogenetic analysis, etc. (Rafalski et. al. 1996).

In recent years, different marker systems such as Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs), Sequence Tagged Sites (STS), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) or microsatellites, Single Nucleotide Polymorphisms (SNPs) and others have been developed and applied to many plant species. Molecular markers can be broadly classified in the following three groups: 1-Hybridization-based DNA markers such as RFLPs and oligonucleotide fingerprinting, 2-PCR-based DNA markers such as RAPDs which can also be converted into sequence characterized amplified regions SCARs, SSRs or microsatellites, STS, AFLPs, inter-simple sequence repeat amplification ISA, CAPS and amplicon length polymorphisms ALPs, and 3- DNA chip and sequencing-based DNA markers such as SNPs (Gupta et. al. 1999). The choice of Molecular markers is objective, convenient and cost dependent. RAPD had already been used to analyze for genetic differences between cultivars of roses. (Debener et al.1996). Molecular markers can be used to verify the origin of vegetatively propagated rose plants of doubtful origin since there is a large number of polymorphism between all varieties of roses investigated and their seedlings (Debener et al. 2000). Two genetic linkage maps of tetraploid roses were generated using AFLPs, Isoyzme, morphological, and SSR markers (Rajapakse et. al. 2001).

SNPs are the most frequently found DNA sequences variations (Kwok, 2001) and they account for 90% of all human DNA polymorphisms (Collins et al. 1998), they also constitute the most abundant molecular markers in the genome and are suitable for automation and can be used for a range of purposes, including rapid identification of crop cultivars, construction of ultra-high-

density genetic maps, and association studies related to genetic disorders (Douabin-Gicquel et al., 2001). Before its use, an SNP needs to undergo detection and validation. Detection can be done experimentally by using DNA sequencing or in silico protocols (Buetow et al., 1999; Sunyaev et al., 1999), both of which are facilitated by an increasing number of EST sequences available in the public domain. Validation can be performed with various techniques, such as primer extension, hybridization, ligation, PCR amplification, and restriction enzyme digestion. Some of the rapid PCR-based assays for validation of SNPs include tetra primer ARMS PCR (Ye, et al., 1992), bi-PASA (Liu et al., 1997), bidirectional AS-PCR (Karhukorpi and Karttunen, 2001), and PCR-CTPP (Hamajima, 2001).

SNPs were studied in several plant species like Barley, Tomato, Wheat, Sugar beet, Soybean, Arabidopsis, rice, Brassica oilseed, and potato. To our knowledge, no studies have been carried out studying SNPs in roses. To understand the genetic basis of key postharvest traits for the development of SNPs markers, expression studies were performed for ethylene biosynthetic enzymes genes, ethylene receptor genes and ethylene signal transduction & transcription genes. The relation of gene expression and sensitivity to ethylene can be further used for developing SNPs molecular markers.

In this study, we hypothesized that:

- The obvious differences in postharvest life between ‘Vanilla’ and ‘Lavender’, cause differences in expression of ethylene receptor genes, ethylene biosynthetic enzymes genes and ethylene signal transduction & transcription genes.
- The highly or lowly expressed genes may contribute to sensitivity or insensitivity to ethylene.

- Differences in expression of ethylene receptor genes, ethylene biosynthetic enzymes genes and ethylene signal transduction & transcription genes between 'Vanilla' and 'Lavender' may be used to develop a specific SNPs Marker of Ethylene sensitivity.

This research had the following two objectives:

- To investigate the expression of ethylene receptor genes, ethylene biosynthesis enzymes genes, signal transduction & transcription genes in both Vanilla and Lavender and the plants from their crossed progenies.
- To develop SNPs marker of ethylene sensitivity, this marker enables rose breeders to select ethylene low-insensitive plants just by running PCR and treating the PCR product with the specific restriction enzyme.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Plant material

Plant material used in this research consists mainly of two-miniature rose's cultivars; 'Vanilla' and 'Lavender', and their offspring's. 'Vanilla' and 'Lavender' and their F1 plants were obtained from Rosen Kordes, W. Kordes' Söhne Rosenschulen GmbH & Co KG, Klein Offenseth-Sparrieshoop, Germany. F1 plants which used in this research were selected depending on the experiments conducted by Ahmadi et al. (2009), in which the F1 plants are subjected to exogenous ethylene treatment, several parameters such as floral buds, flower and leaves abscission were measured. Eight F1 plants were selected; the first 4 genotypes (128, 48, 131, and 67) considered as low sensitive to ethylene and the other 4 genotypes (50, 74, 143, 22) considered as high sensitive to ethylene. Two genotypes of each group were used for expression analysis (131, 48) and (50, 74). Plants that resulted from self-pollination of 'Vanilla' and self-pollination in 'Lavender' were also studied, more details of the plant material used in this research are provided in Table 1. The plants were grown in the greenhouses of Institute of Floriculture, Tree Nursery Sciences and Plant Breeding, University of Hannover under the following conditions: 22°C day / 20°C night temperatures and 60-85% relative humidity with a supplement of natural daylight with 60 μmol m^{-2} s^{-1} from SON-T lamps (Osram, 400W, Philips, Eindhoven, The Netherlands) over a 16 h photoperiod. Plant samples from required plants cultivars and stage of development or organs were collected and immediately kept in liquid nitrogen then they have been ground to a fine powder under liquid nitrogen using a pre-chilled mortar and pestle and stored in a -80°C freezer until use for either RNA or DNA extraction. For genomic southern blot hybridization, DNA from 'Vanilla' along with DNA from *Rosa multiflora* hybrid were used.

Table 1**. Special characters of Rosa hybrida L. cultivars used in this study.**

Cultivar	Sensitivity to exogenous ethylene treatment
Parents	
1- Vanilla	Low
2- Lavender	High
F1	
1. 48	Low
2. 128	Low
3. 67	Low
4. 131	Low
5. 50	High
6. 74	High
7. 142	High
8. 22	High
S1 Lavender	
1. LxL 89	Low
2. LXL 72	High
S1 Vanilla	
1. VXV 01	Not studied

2.2 Crossing experiments

Several crosses were performed during the research period, these crosses are:

1. Self-crossing of 'Vanilla'.
2. Self-crossing of 'Lavender'.
3. Back-crossing of 'Vanilla'.

The above crosses were carried out in the above-mentioned greenhouse conditions during the whole year and whenever the plants produced flowers, extensive crossings were done in the spring season. Briefly, flowers, just before opening, were emasculated in case of backcrosses, pollinated and covered with paper bags to prevent pollination from other plants in greenhouse. Anthers were obtained from pre-open stage flowers in the morning, they were collected by detaching the mature

anthers in a 9 cm diameter Petri dishes and leaving in silica gel desiccators overnight. Pollen grains were then collected and kept in refrigerator for short storage up to 1 week or in -80 freezers for long storage period.

2.3 Pollen germination test

Pollens from 'Vanilla', 'Lavender' were tested for viability and ability to germinate in vitro on a medium containing H_3BO_3 (40 mg/l), $CaCl_2.6H_2O$ (226.5 mg/l), sucrose (150g/l), and agarose (7 g/l). The pH of this medium had been adjusted to 5.6 with 0.1 N NaOH before the addition of agarose and autoclaved at 110 °C for 10 minutes (Gudin et al. 1991). After cooling to 55 °C the medium was poured in 6 cm-diameter sterilized petri dishes (5 ml per dish). The dishes then were sealed with parafilm and stored in darkness at lab temperature not more than 2 weeks. Full open flowers from each cultivar were collected and the anthers were detached directly into 9 cm-diameter petri dishes. The petri dishes then kept in silica gel desiccators for 1-2 days until the anthers release the pollen grains. Releasing the pollen grains was assisted by hand shaking of closed petri dishes, pollens were then scattered over the germination medium using paint brush and kept overnight in dark at room temperature. The germination percentage was calculated by counting the pollen grains in the area of randomly selected microscopic view, at least 150 grains were enumerated. The pollen grain that germinated at least as long as the grain diameter was considered as germinated one.

2.4 DNA Isolation

Genomic DNA was isolated from young leaves using two methods depending on the concentration required. For PCR optimization conditions a modified method of Edwards et al., 1991 were used; in which, 100 mg of plant tissues were ground in an Eppendorf tube by Polypropylene tissue grinder attached to Homogenisator in presence of 400 µl of extraction buffer (200mM Tris-Hcl pH 7.5, 250mM NaCl, 25mM EDTA, 0.5 % SDS). Then the samples were vortexed for 5-10 seconds, then

they were incubated 15 minutes in water bath at 65 °C, after that 200µl of 5M Calcium acetate was added to each sample, the sample were then incubated on the ice bath for 10 min. after that they are centrifuged at 13,000 × *g* for 20 minutes at room temperature and 500µl of the supernatant transferred to a new Eppendorf tube. This supernatant is mixed with 500µl pre-chilled isopropanol and left at room temperature for 5-10 minutes. Then they were centrifuged at 13,000 × *g* for 10 minutes at room temperature. Then the DNA pellets were washed with 1ml 70% ethanol. Then they left for 15-30 minutes to dry and finally dissolved in 50µl dd-water and kept in -20 °C freezer. For southern blot analysis DNeasy Plant Maxi Kit (Qiagen, USA) was used to isolate high yield of DNA as described by the manufacture with some modifications; 2.0 g (fresh weight) was used as starting plant material and the elution step was done 4 times. The elution time was extended up to 30 min. and done with 750µl. Then all isolations were mixed together and ethanol precipitated by adding 1/10 volume 3M sodium acetate pH 5.2 and 3 volumes cold 100% ethanol, left at -20°C for at least 30 min. then centrifuged at 4 °C for 20 min. at 14000 × *g*. The pellet then washed with 75% Ethanol, centrifuged at 4 °C for 5 min. at 14000 × *g*, and left under hood to dry for approx. 20 min. Finally, the pellets were resuspended in 100µl dd-water.

2.5 RNA Isolation

Total RNA was isolated from 'Vanilla' and 'Lavender' and their progenies. RNA from three different developmental stages (Figure 2) of petals and pistils was isolated along with RNA from floral buds, and leaves. In this research two methods of isolation were used; when only low yield RNA is required or low mount of starting plant material is available, Invisorb® Spin Plant RNA Mini Kit (Invitek, Berlin, Germany) was used as described by the protocol supplied by the manufacture. A modified hot borate method (Wilkins and Smart, 1996) was used to isolate high yield of good quality RNA. Two grams of finely ground, frozen plant material were mixed with

8ml of the pre-heated 80-90°C extraction buffer (1% SDS, 1% sodium deoxycholic acid, 5mM EDTA, 0.2M Borax (Sodium borate decahydrate)) in 50ml Oakridge tubes. PVP 2% DTT 1%, Np-40 1% were added to the extraction buffer just before heating. 110µl of Proteinase K (20mg/ml) was added to each tube. The tubes were then incubated at 42°C for 90 minutes in water bath, during the incubation time, the samples were vortexed each 10 minute. Then the tubes were removed from water bath and immediately 1040µl 2M potassium chloride was added (to make the final concentration of 160mM KCl) and the tubes were shaken vigorously and incubated 1 hour on ice. After that, the tubes were Centrifuge at 10.000 × g for 20 minutes at 4°C. After centrifugation, the aqueous phase was transferred to a new, labeled 20 ml tubes and 1/3 volume of 8M LiCl was added to a final concentration of 2M LiCl. Then the tubes were placed in refrigerator (4°C) overnight. The following day RNA was precipitated by centrifugation at 14.000 × *g* at 4°C for 20 min. The supernatants were decanted and discarded, and the pellets were washed in 4ml of 2M LiCl, the pellets were dispersed and dissolved with mildly shaking or with a sterile disposable pipette tip as needed. the wash step was repeated 3 times at least, After 3 times of washing, the supernatant should be colorless, if not, the pellet was washed again till the supernatant becomes colorless. Then the pellets were suspended in 2ml of 10 mM Tris-HCl (pH 7.5) through gently vortexing, then the insoluble materials were removed from the RNA pellet by centrifugation at 10.000 × *g* for 10 minutes at 4°C. The supernatants decanted into new 20 ml pre-labeled tubes and 1/10 volume 2M potassium acetate (pH 5.5) was added to each tube and incubated on ice for 15 minutes to remove positively charged polysaccharides, residual proteins, and other salt-insoluble materials. The tubes were then centrifuged at 10.000 × *g* for 15 minutes and the supernatants were decanted again into new 20 ml pre-labeled tubes. 2.5 X volume of cold 100% ethanol and 1/10 volume of 3M sodium acetate (pH 6) were added to the tubes and gently mixed by inverting the tubes, then the precipitation of RNA was allowed to take place overnight at -20°C, next day the tubes were

centrifuged at 16.000 × *g* for 30 minutes at 4°C. The supernatants were then discarded and the pellets were washed with 2ml 70% ethanol and centrifuged at 16.000 for 5 minutes at 4°C. The ethanol was discarded and the pellets were allowed to dry for 15-30 minutes inside the laminar airflow and finally the pellet was dissolved in 300 µl DEPC-treated water.

Figure 2.
Three flower stages of each 'Vanilla' and 'Lavender'.1: pre-blooming stage, 2: full blooming stage, 3: post blooming stage.

2.6 DNA & RNA Quantification

DNA & RNA concentration was measured spectrophotometrically using SmartSpec3000 (Bio-Rad, USA). To confirm the RNA & DNA quantity and quality, they electrophoresed on a 1% agarose gel with 0.3 µg/ml ethidium bromide and compared with standard concentrations (10, 25, 50, 100, 200, and 300 ng) of λDNA (Fermentas GmbH, Germany) and then visualized by using a BioDocAnalyze UV transilluminator (Biometra, Germany).

2.7 Database searches & bioinformatic software

Computer searches for gene sequences were performed using the NCBI (National Center for Biotechnology Information) site (http://www.ncbi.nlm.nih.gov/) which includes also BLAST software (Basic Local Alignment Search Tool) (NCBI; Altschul et al., 1997) which used for sequence similarity searching and identifying genes and genetic features. The primers were

designed by using of the online version of Primer 3 software (Rozen and Skaletsky, 2000; http://frodo.wi.mit.edu/primer3/input.htm). Sequences were edit, viewed, and analyzed by several software including Chromas software (http://www.technelysium.com.au/chromas.html), DNAClub and Genamics Expression (http://www.genamics.com/expression/). The sequences were aligned and analyzed using online version of ClustalW software, European Bioinformatics Institute (EMBL; Higgins, 1994). The restriction map of the PCR products was obtained either with in silico simulation of molecular biology experiments software (http://insilico.ehu.es/) or NEBCutter V2.0 (http://tools.neb.com/NEBcutter2/index.php).

2.8 Primer construction & optimization

Oligonucleotide primer pairs that used in this study were summarized in table 2. Gene specific primers for PCR (Table 2) were designed using primer3 software (section 2.7). The primers were designed to produce amplicons from 100–800 bp. The oligonucleotides were synthesized by MWG Biotech AG (Ebersberg-Munich, Germany). To obtain stronger amplification rate, annealing temperature of each primers pairs were tested by using with a range of +4 °C to -6 °C of the calculated melting temperature (Tm). The fragments of target genes were amplified in 20µl reaction volume containing 10.4 µl of sterile water, 10mM Tris-Cl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.001% gelatin, 150 µM each of dATP, dCTP, dGTP and TTP (fermentas), 0.25 pmol/µl of each primer, 0.5 unit of Axon taq DNA polymerases (Axon Labortechnik, Kaiserslautern, Germany), and 40-100 ng of genomic DNA. DNA amplification reactions were performed in a Hybrid Thermal cycler with temperature programming as follows: an initial denaturation step at 95°C for 5 min, 36 cycles of denaturation at 95°C for 30 s, annealing at 50-62°C (Table.2) for 60 s and extension at 72°C for 2 min., and a final extension at 72°C for 10 minutes. The PCR products

were analyzed by gel electrophoresis on 1% (w/v) agarose gel and DNA bands were visualized with ethidium bromide by using a BioDocAnalyze UV transilluminator (Biometra, Germany).

Table 2. Specific primer pair of Ethylene receptor genes, ethylene biosynthesis and ethylene signal transduction & transcription gene

Acc. No.	Primer Name	Sense PRIMER 5'-3'	Antisense PRIMER 5'-3'	Genomic size (bp)	mRNA Size (bp)
AF394914	*RhETR1*	TGGTATGAACCTTCAACTTTCTCA	CGCATAGACTCTTCAAGAATAGCA	521	393
AF127220	*RhETR2*	CTCAAACTTCCAAATCAATGACTG	ATATTCTGCTCCATTAGCAGATCC	978	213
AF154119	*RhETR3*	CACTGCTATAACGCTCATCACTCT	CATTAGTTGGGACTCTTCAAGGAT	661	661
AF159172	*RhETR4*	TTTGAATCTGCAACTTTCTCACAC	GCATTTCGTGGTTCATGACAG	500	500
AY061946	*RhACS1*	AAACGTCACCGTTCCAACTC	CTGAATTTCCGATGGCCTTA	205	205
AY525066	*RhACS2*	AAAAACCCAGAAGCCTCCAT	AAGGAACGGGAACCAGAAAT	370	250
AY525067	*RhACS3*	CCATGGCCTTTTGTCCTTTA	GGGTTGGAGGGGTTTGTAAT	126	126
AY525068	*RhACS4*	GCTTCCAACTTGGGATCAAA	TGGGGTTGGAACTAGCAAAG	237	237
AY525069	*RhACS5*	CAGCCGGATTCAAGAGAAAC	GGCGAGGCAAAACATAAGAG	203	203
AY032953	*RhCTR1*	GGCTCTGATGTTGCTGTGAA	TCAATGGCCTCAAAGATTCC	706	706
AY029067	*RhCTR2*	TTCCTTCCAAGGGGAAGTCT	CCCACTCCAAGCCAATTTTA	375	375
AF443783	*RhEIN3*	CCCTGCAGCCATAGACAAGT	ACCCTGATTTCATCCACCAA	236	236
AY052825	*RhEIL*	TCCCTGGTTTGATGGAAGAC	GAGGCCACCATTCCTCATTA	192	192
BI977396	*Rh-ß-Actin*	CCATGTTCCCTGGTATTGCT	GCCTTTGCAATCCACATCTA	525	395

2.9 DNAse I treatment

DNase I treatment was performed to degrade genomic DNA contaminating RNA preparations; 1µg RNA is mixed with 1 unit of DNase I RNase-free in presence of Reaction Buffer (10 mM Tris-HCl (pH 7.5), 2.5 mM MgCl2, 0.1 mM CaCl2) and 20 units of 0.5µl RNase inhibitor (Fermentas GmbH, Germany) and incubated at 37°C for 30 minutes in T3 thermocycler (Biometra, Germany). The reaction was stopped by addition of 5mM EDTA and heating to 70°C for 10 minutes. To prove DNA degradation from RNA samples, to test the RNA quality after DNase treatment, and to test the deactivation of DNase I, 1µl from each RNA sample that treated with DNase I was taken and mixed with 50ng of λDNA in a total volume of 10µl and incubated for 30 min at 37°C and compared with 1µl from the same sample without treating with λDNA by running on same 1% agarose gel containing 0.3 µg/ml ethidium bromide.

2.10 cDNA synthesis

The first strand complementary DNA (cDNA) was prepared using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant kit (Promega, USA). One microgram of total RNA was mixed with 1µg of the Oligo dT (23) primer in a total volume of 7 µl. The mixture then was heated to 70°C for 5 minutes using the T3 thermocycler (Biometra, Germany) and quickly cooled on ice for 5 minutes. Then the following components were added to the annealed primer in this order: M-MLV RT Reaction buffer (50 mM Tris-HCl (pH 8.3), 75mM KCl, 3 mM MgCl2, 10 mM DTT) (Promega, USA), 0.5 mM dNTP, 100 units M-MLV RT (H-) (Promega, USA), 20 units RNase out (Fermentas GmbH, Germany) in total volume of 12.5µl. The reaction then was incubated for 10 min at 40°C, then for 150 min at 50°C and then for 15 min at 70°C as a final deactivation step of transcriptase.

2.11 RT-PCR

Two steps Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed in which total RNA was reverse transcribed to cDNA as indicated in section 2.10 as the first step and PCR were performed using the resulted RT reaction (cDNA) as the second step. A pair of primers was constructed to amplify the internal control rose Beta-actin gene (Rh-ß-actin) cDNA based on the reported mRNA sequence of *Rosa chinensis* (Table 2). To analyze transcript levels of the investigated genes, amplification of the Rh-ß-actin has been optimized to have nearly similar amplification by altering the concentration of template cDNA. To determine the cycle numbers for RT-PCR, serial cDNA dilutions were used as template for RT-PCR using different cycle numbers (20, 25, 30, 35, 40, and 45 cycle), the PCR products obtained with 45 cycles were the maximum and with some little differences among the PCR products resulting from amplification of different cDNA concentrations. Therefore, the RT-PCR was run for 30 cycles in next experiments because at this cycle, the maximum differences in amplification as expected from the different cDNA concentrations were obtained. Because ß-actin should express similarly in all tissues, it is assumed that the differences in expression among different cDNA was due to cDNA concentration, therefore cDNA concentration was altered from all tissues investigated to have same or nearly same transcripts of ß-actin to ensure that an equal amount of cDNA had been added to each PCR reaction. Second-Strand cDNA Synthesis and PCR Amplification was performed in total volume of 20µl as previously described in section 2.8 except that 2 µl of cDNA were used instead of genomic DNA. RT-PCR reaction was conducted in T3 thermocycler (Biometra, Germany) under the following conditions: an initial denaturation step at 95°C for 2 min, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50-62°C (Table 2) for 40 seconds and extension at 72°C for 2 min., and a final extension at 72°C for 10 minutes. PCR products were electrophoresed as described in section 2.8. As a negative control for RT, a mixture of different randomly chosen RNA samples

were subjected to the same reaction without reverse transcriptase. All RT-PCR experiments were repeated at least twice.

2.12 Cloning of the PCR products

The amplified products were purified directly after PCR or after cut from agarose gel by using The Invisorb® Fragment CleanUp (Invitek, Germany) as described by manufacturer. The RT-PCR products (cDNA fragments) were cloned for sequencing and subsequent analysis using pGEM-T easy vector before being transformed into *Escherichia coli* DH5α-competent cells according to the manufacturer's instructions (Promega Corp., Madison, WI) with some modifications. Briefly, 20-100ng of the PCR product was mixed with Rapid Ligation Buffer, 25ng pGEM®-T Easy Vector, and T4 DNA Ligase. The final reaction volume for ligation was 5 µl. 25 ng of vector was used with the molar ratio of insert to vector being set at 3:1. The ligation mixture was allowed to ligate overnight at 4°C in the refrigerator. 2 to 5µl of ligated mixture were used for transformation into E. coli DH5α-competent cells. After that 250 µl of room temperature S.O.C. medium (2%Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose) was added to transformation reaction and incubated in the shaker at 180× g for 60-90 minutes at 37°C. The transformation culture then was cultured onto LB (1% Tryptone, 0.5% Yeast extract, 0.5% NaCl, pH 7.0) medium plates with ampicillin (100µg/ml), IPTG (0.5 mM), and X-Gal (80µg/ml) and incubated at 37°C overnight.

To allow identification of inserts prior to sequencing, about ten different white colonies were randomly picked with sterile toothpick, sub-cultured onto another properly labeled LB plate by streaking in 2 parallel lines and immediately dipped in the PCR tube and mixed vigorously with PCR reaction. The selected colonies were amplified in 20µl reaction volume as exactly as described in section 2.8 except that the sterile water volume was increase to 12.4 µl. The primers were specific

to the pGEM-T easy vector and consist of pGEM-T forward primer $^{5'}$TAAAACGACGGCCAGTGAAT$^{3'}$ and reverse primer $^{5'}$CTCAAGCTATGCATCCAACG$^{3'}$ with a flanking region of 175 bp.

2.13 Isolation of plasmid DNA & Sequencing

After confirmation of insert presence in the selected colonies by PCR, each single colony was grown overnight at 37°C on a shaker in 5 ml liquid LB medium supplemented with 100µg/ml of Ampicillin. Cells were centrifuged at 10,000 × *g* for 5 min. Plasmid DNA was isolated using Wizard® Plus SV Minipreps DNA Purification System Kit (Promega, USA) according to the manufacturer's protocol. Sequencing was carried out commercially by MWG Biotech AG (Ebersberg, Germany) using SP6 or T7 primers. Further sequence analysis was performed by several software including Chromas, DNAclub, Genamics Expression, ClustalW, and BLAST as described in section 2.7

2.14 Northern blot hybridization

2.14.1 Digoxigenin labelled probe synthesis

Non-radioactive molecular probes were generated by means of the DIG DNA Labeling Mix. (Roche Diagnostics GmbH, Mannheim, Germany) in which a digoxygenin labeled probes by PCR were synthesized. Briefly, the fragments of target genes were amplified in 30µl reaction volume containing 16.2 µl of sterile water, 10mM Tris-Cl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.001% gelatin, 1.5 µl of the DIG DNA Labeling Mix (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM DIG-dUTP, alkali-labile, pH 7.5 (20°C), 0.25 pmol/µl of each forward and reverse primer, 1.5 units of Axon DNA polymerases (Axon Labortechnik, Kaiserslautern, Germany), and 20-60pg plasmid DNA that contains the target gene. DNA amplification was performed as

described in section 2.8. To test the labeling quality, a control reaction was carried out along with the labeling reaction but with the use of normal dNTPs instead of DIG DNA Labeling Mix. Due to multiple incorporations of DIG-dUTP during the PCR process, the molecular weight of the PCR products is significantly increased compared to the unlabeled PCR product. The DIG-labeled probes were denatured at 95°C for 10 min and immediately chilled on ice for at least 3 minutes before mixing with Hybridization buffer. The DIG labeled probes were used for Northern blot hybridization and Southern blot hybridization. After hybridization is completed the probes were stored at -20°C until the next usage.

2.14.2 Preparation of RNA samples & formaldehyde-agarose gel

Total RNA was isolated as described in section 2.5, 10 µg of total RNA were mixed with 20 µl of freshly prepared RNA denaturation loading mixture (Table 3) in a final volume of 30 µl, and then the sample were heated for 15 min at 65°C, and immediately placed on ice for at least 2 minutes or until loading onto the formaldehyde-agarose gel (Table.3). All materials required for gel preparation were cleaned with RNase-ExitusPlus™ (Applichem, Darmstadt, Germany) followed by washing with DEPC-treated water to eliminate any sources of RNase. The denatured RNAs were then separated on 1% denaturing formaldehyde-agarose gel in 1x MOPS-buffer in Sub-Cell GT DNA Electrophoresis Cell (Bio-Rad Laboratories Inc.) at constant voltage of 4V/cm gel length for 4-5 h.

2.14.3 RNA blotting & crosslinking

To assess the quality of RNA separation, the gels were visualized under UV transilluminator and photographed. The gels were washed once in DEPC-treated water for 15 min and then two times in 10x SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.5) for 15 min each. Separated RNAs were transferred to Hybond-N+ nylon membranes (Amersham, GE Healthcare

UK limited, Buckinghamshire, UK) with a vacuum blotter (Model 785 Vacuum Blotter, Bio-Rad Laboratories Inc.) according to the manufacturer's protocol in 10xSSC. After transferring RNA to the membrane, Gels were viewed under UV transilluminator to confirm RNAs transfer onto the membrane. RNAs were immobilized on the membrane by UV crosslinking (CleneCab Plus, Herolab GmbH, Wiesloch, Germany). The membrane was exposed to 70.000 µjoules/cm^2 energy as recommended by manufactured company. Membrane was rinsed with DEPC-treated water and left to dry at room temperature or used directly for hybridization.

Table 3. Solution and buffers used in Northern and Southern blot hybridization.

Solution	Components	Final Concentration	Notes
DIG DNA Labeling Mix, 10 × conc.	1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM DIG-dUTP, alkali-labile, pH 7.5(20°C)	0.05 mM dATP, dCTP, dGTP, 0.0325 mM dTTP, 0.0175 mM DIG-DUTP, alkali-labile	
DEPC-treated water (1 liter)	1 ml DEPC 1 liter of water	0.1% DEPC	Stir strongly 2-3 h and incubate at 37°C overnight, and autoclave.
1 % Agarose - Formaldehyde-MOPS gel (300ml)	3.0 g Agarose 17.5 ml Formaldehyde 30.0 ml 10X MOPS 250 ml DEPC water 10 µl Ethidium Bromide	1% Agarose 0.7 M formaldehyde 1X MOPS 0.5µg/ml Ethidium Bromide	Preheat Formaldehyde and MOPS at 55°C. Heat the mixture to melt agarose. Cool to 55°C in a water bath The gel should be cast, loaded, and run in the hood.
RNA denaturation loading mixture	10 µl RNA 83 µl Formaldehyde 37% 250µl Formamide (deionized) 50µl 10X MOPS buffer 10 µl Bromophenol 2.5% (w/v)	10µg RNA 0.7 M 50% 1X 0.05 % (w/v)	Make always fresh
1X RNA Electrophoresis buffer	200 ml 10X MOPS 1800 ml DEPC water	1X MOPS	
10X MOPS (pH 7.0) (1 liter)	41.8 MOPS 6.8 Sodium Acetate 20ml of 0.5 M EDTA 800ml H2O	200 mM MOPS 50 mM Sodium Acetate 10 mM EDTA	PH to 7.0 w/ NaOH, raise vol. to 1 liter. Autoclave and store in the dark at RT. It is normal for 10x MOPS to turn a little yellow after it is autoclaved.
20X SSC (standard saline citrate) (pH 7.0) (1L)	175.3 g NaCl 88.2 g Na-citrate	3M NaCl 0.3M Na-citrate	Adjust PH with 37% HCl, then autoclave
10% SDS (1L)	100 g in DEPC-treated water	10% SDS	
Maleic Acid Buffer pH 7.5 (1L)	11.61g Maleic Acid 8.766g NaCl	0.1M Maleic Acid 0.15M NaCl	adjust pH with NaOH pellets (≈7g)

low stringency buffer (1L)	100 ml 20x SSC 10 ml 10% SDS 890 ml DEPC-treated water	2xSSC/0.1%SDS	
high stringency buffer (1L)	25 ml 20x SSC 10 ml 10% SDS 965 ml DEPC-treated water	0.5x SSC/0.1% SDS	
Washing buffer (1L)	Maleic acid buffer 750 ml + 2,25 ml Tween 20		
10X blocking stock solution	25 ml Blocking-stock-solution to 225 ml Maleic acid buffer		Preheat 100ml maleic acid buffer at 60°C. add 10g blocking reagent (Roche) and stir 1 h. autoclave.
1x blocking solution (250 ml)	25 ml 10X Blocking stock solution 225 ml Maleic Acid Buffer		
Anti-Dig-AP solution	2.5 µl Anti-Dig-AP 50 ml 1X blocking solution	1:10 000 (75 mU/ml) in Blocking Solution	
Detection buffer (0.5L)	50 ml of 1M Tris-HCl 10ml of 5M NaCl	0.1M Tris-HCl pH 9.5 0.1M NaCl	
Substrate dilution buffer (0.2L)	2.12g Diethanolamine 0.04g MgCl2	0.1 M Diethanolamine 1 mM MgCl2	2.12g in 160ml water, adjust pH to 9.5, add 0.04 g $MgCl_2$, add water to a final volume of 200ml
Stripping buffer (Northern) (1L)	5 ml of 20x SSC 50 ml of 10% SDS 945 ml DEPC-treated water	0.1xSSC, 0.5% SDS	
Stripping buffer (Southern) (1L)	20 ml of 1M NaOH 10 ml of 10% SDS 970 ml of ddH_2O	0.2 M NaOH containing 0.1% SDS	

2.14.4 Hybridization of Probe & Detection

Pre-hybridization and hybridization was carried out in rotating hybridization oven (ProBlot Hybridization Systems, Labnet International, NJ, USA). The membranes were inserted in hybridization tubes containing 15 ml of Dig Easy Hyb (Roche Diagnostics GmbH, Mannheim, Germany) at 50°C for at least 30 min. Hybridization of RNAs to the DIG labeled probe took place overnight under the same conditions with 100-150ng of denatured DIG-labeled probe in 7 ml of

Dig Easy Hyb. The post-hybridization washes were performed in the following order: 1) twice for 5 minutes each in low stringency buffer at room temperature, 2) twice for 15 minutes each in high stringency buffer at 50°C, 3) once in washing buffer for 2 minutes at room temperature. The membrane was then agitated in 1x blocking solution (Roche Diagnostics GmbH, Mannheim, Germany) for 30 minutes to suppress nonspecific hybridization. Alkaline phosphatase anti-digoxigenin conjugate (1:10000) (Roche Diagnostics GmbH, Mannheim, Germany) was then added and the membrane incubated for 30-60 min at room temperature. The membrane was then washed two times for 15 min each with washing buffer and finally for 2-5 min in detection buffer. The chemiluminescent reaction was performed with CDP-star (Roche Diagnostics GmbH, Mannheim, Germany) as a substrate and detected with Amersham Hyperfilm ECL (Amersham, GE Healthcare UK limited, Buckinghamshire, UK) in x-ray cassette to the membranes for different period of time depending on signal and background ranging from 5 minutes to 3 hours. The transcript sizes were estimated by comparison with the RNA Molecular Weight Marker I (DIG-labeled, 0.3–6.9 kb; Roche, 100ng). The membrane was stripped by boiling in stripping buffer, followed by re-hybridization.

2.15 Southern Blot Hybridization

2.15.1 Gel Preparation and Blotting

Genomic DNA was extracted from the leaves of 'Vanilla' and diploid roses as described in section 2.4, Approximately 10 µg of DNA per sample was digested with EcoRI, EcoRV, HindIII and XbaI (2 units/µg) (MBI Fermentas) and incubated at 37°C overnight. The digested DNA was electrophoresed on a 0.8% agarose gels in 1x TAE buffer at 30V overnight. Following electrophoresis, the gel was visualized under UV transillumination (BioDocAnalyze, Biometra, Germany) to confirm DNA separation and photographed. The DNA was nicked by depurination in

0.25M HCl for 10 minutes at room temperature with gentle agitation, then soaked in denaturation solution (0.5M NaOH. 1.5M NaCl) two times each for 15 minutes at room temperature, then soaked in neutralization solution (0.5M Tris-HCl; pH 7.5, 1.5M NaCl) two times each for 15 minutes at room temperature, and finally equilibrated in 20X SSC for 10 minutes at room temperature with gentle agitation. The gel was rinsed in distilled water between each step. DNA blotting, crosslinking, pre-hybridization, hybridization, washing, and detection were performed as indicated in section 2.2.7.3-4., except that the prehybridazation and hybridization temperature was 42°C. The same Digoxigenin labelled probes, which were prepared for Northern blot, were used to hybridize DNA in southern blot. The membrane was stripped by washing two times in stripping buffer (0.2M NaOH, 0.1% SDS) each time for 20 minutes at 37°C followed by rinsing in 2X SSC for 5 minutes at room temperature. The membrane was allowed to air dry at room temperature or used directly for hybridization. Sizes of resulting hybridization bands were estimated by comparison with the DNA Molecular Weight Marker III (DIGlabeled, 0.56–21.2 kb; Roche, 1µg).

2.16 Reverse northern dot blot

Reverse Northern dot blot was used to confirm the differences in expression of the cDNA fragments of ethylene biosynthetic enzymes genes, ethylene receptor genes and ethylene signal transduction & transcription genes.

2.16.1 Synthesis and labeling of first-strand cDNA probes

DIG-labeled first strand cDNA probes were prepared by reverse transcription of total RNA in 40µl reactions. Briefly, 25µg total RNA, 2µg oligo$(dT)_{15}$, and 3µg random hexamers were mixed in a total of 20µl, heated to 70°C for 10 minutes, then placed immediately on ice for 5 minutes. The RNA-primer reaction was mixed with M-MLV RT Reaction buffer (50 mM Tris-HCl (pH 8.3), 75mM KCl, 3 mM MgCl2, 10 mM DTT) 0.25 mM of dATP, dCTP, dGTP, and 0.16 mM dTTP,

0.05 mM Digoxigenin-dUTP, and 300U M-MLV Reverse Transcriptase (H-) (Promega, USA) in total volume of 20µl. The mixture was incubated at 40°C for the first 10 minutes and at 50°C for the last 50 minutes. Heat inactivation at 70°C for 15 minutes was performed to terminate the reaction. The probes were treated with 2U of RNase H for 20 min at 37°C, purified using Invisorb® Fragment CleanUp (Invitek, Germany), and heat-denatured for 10 min at 95°C and immediately cooled on ice before use.

2.16.2 Preparing dot blot

Dot blot is prepared by manually spotting 2 µl of purified and denatured (95°C for 5 min) PCR-amplified cDNA fragments in 3 different dilutions (1:10, 1:50, 1:100) onto a duplicate positively charged nylon membrane (Hybond N+ membrane (Amersham). PCR products from different investigated genes were amplified from cDNA as described in section 2.7. Template cDNA was diluted 1:10 before using in PCR reaction. PCR conditions were 94°C for 2 min (initial denaturation) followed by 40 cycles of 94°C (denaturation) for 30 sec., primer specific annealing temperature for 1 min and 72 °C (extension) for 2 min and a final extension of 72°C for 5 min. Each PCR product was precipitated with 1/10 volume 3M sodium acetate pH 5.2 and 3 volumes cold 100% ethanol and re-suspended in 2µl dd-water. The membrane was soaked in denaturing (0.5 M NaOH, 1.5 M NaCl) and neutralizing (0.5 M Tris-HCl pH 7.5. 1.5 M NaCl) solution for 5 min each successively, the membrane was washed briefly in 2x SSC and cut into two identical parts and the cDNAs were crosslinked to the membrane with a UV crosslinker (CleneCab Plus, Herolab GmbH, Wiesloch, Germany). The dot blot was then prehybridized, for at least 30 min with the prehybridization buffer (Dig Easy Hyb, Roche Diagnostics GmbH, Mannheim, Germany) at 42°C and subsequently hybridized overnight at 42°C with the DIG Labeled cDNA probe. Washing, blocking, and detection steps performed as described above in section 2.14. The membrane was

stripped by washing two times in stripping buffer (0.2M NaOH, 0.1% SDS) each time for 20 minutes at 37°C followed by rinsing in 2X SSC for 5 minutes at room temperature. The membrane either allowed to air dry at room temperature or used directly for next hybridization.

2.17 Identification of SNPs & CAPS

In order to identify single nucleotide polymorphisms, the PCR products from differentially expressed genes were cloned and sequenced as described above. Sequences from 'Vanilla' and 'Lavender' were compared for homology by using ClustalW (http://www.ebi.ac.uk/clustalw/). To validate the potential SNPs by CAPS marker, the SNP would have to be in a restriction enzyme recognition site. The restriction map of the PCR products was obtained either with online software of In silico simulation of molecular biology experiments or NEBCutter V2.0 software as described in section 2.7. to assist the development of set of CAPS markers that used to distinguish the different alleles of the investigated genes. DNA amplification and the PCR condition were carried out as described in section 2.8. PCR products were digested with prober restriction enzymes by mixing 10µl PCR product with 10 µl of appropriate restriction buffer containing 0.5 unit of restriction enzyme. The digests were then incubated at the temperature recommended by the manufacturer for overnight. CAPS polymorphisms were then analyzed by electrophoresis on 2% agarose gel containing 0.3 µg/ml ethidium bromide in 1 x TAE buffer. Gene Ruler DNA ladder Mix (Fermentas) was used as a size marker.

2.18 Densitometric analysis of RT-PCR results

Images were analyzed to quantitative the amount of hybridization of each sample, using ImageQuant software (Amersham, GE Healthcare UK limited, Buckinghamshire, UK). Data were further analyzed with Microsoft Excel and SAS software packages. The RT-PCR results from electrophoresis gels were digitalized and converted to numbers. For these cases, the method was

applied semi-quantitatively: the optical density of each amplified band was calculated using the ImageJ image processing program and numerically expressed as the relative density in comparison to the optical density of the background. All factors that could influence these measurements (PCR conditions, number of amplification cycles, and thickness of the agarose gel, image capture and scanning procedures) were standardized to avoid systemic errors. Furthermore, all results were normalized to the expression of the housekeeping gene ß-actin, which is constitutively expressed in all cells and serves therefore as an internal standard (Thellin et al., 1999). Under these conditions, gross quantitative estimations were possible and broad differences in mRNA expression could be detected.

CHAPTER 3

RESULTS

3. RESULTS

3.1 Crossing Experiments:

Among hundreds of self-crosses of 'Vanilla', only one plant was obtained during the period of study, but after investigating the reasons that prevented the success of self-crossing in 'Vanilla', many hips were harvested and now in process of germination. Self-crosses of 'Lavender' were successful and many hips were harvested. After germination, we got many plants, which were investigated by another researcher for exogenous ethylene treatment.

Backcrossing of 'Vanilla' was successful for one genotype (42/131). Other selected genotypes were either not successful or produced no pollen grains.

3.2 Pollen Germination test

Preliminary investigation of in vitro germination of 'Vanilla' pollen revealed that germination percentage and the growth of pollen tubes on the medium indicated in section 2.3 was always poor. In last spring season, some of some very young plants produced very good-looking flowers with high number of pollen grains. Those pollen grains were collected over the period of time and subjected to in vitro germination test, the results showed that germination percentage and the growth of pollen tubes was higher compared to pollen grains obtained from other 'Vanilla' plants (Figure 3).

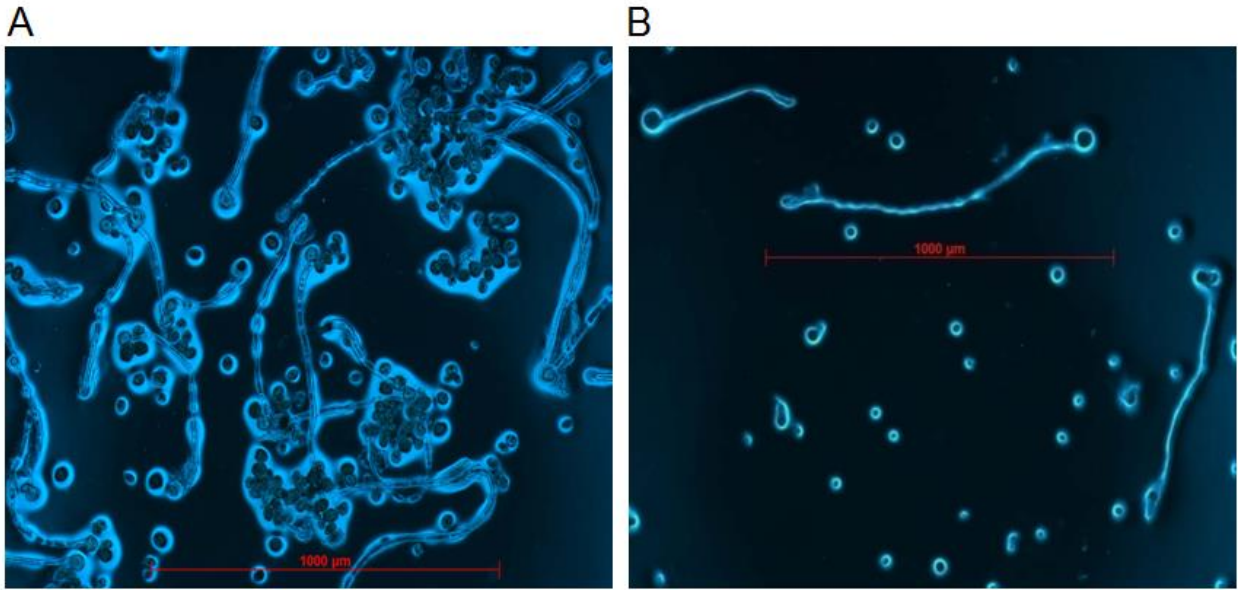


Figure 3.
In vitro pollen germination pollen tube growth of 'Vanilla' after overnight incubation in dark, high quality pollen (A) low quality pollen (B).

3.3 Expression Studies

3.3.1 Optimization Cycle number

Due to the fact that the PCR product can attain a plateau during the latter stages of the reaction, the suitable number of PCR cycles has to been determined to cease the PCR reaction before it reaches this plateau. The appropriate number of cycles of PCR reaction depends mainly on the starting concentration of template cDNA when other parameters are optimized. An experiment includes five serial dilutions of cDNA and six different cycle numbers particularly 20, 25, 30, 35, 40, and 45 was conducted to amplify *Rh-β-actin* gene. Comparison of the *Rh-β-actin* gene expression showed no considerable differences after 35 cycles of PCR; in contrast, very low PCR products could be visualized below 20 cycles of PCR. Therefore, the optimal number of PCR cycles required to quantify the mRNA expression of Rh-β-actin gene was determined as 30 cycles. Thus, all the next RT-PCR experiments were done on that basis (Figure 4).

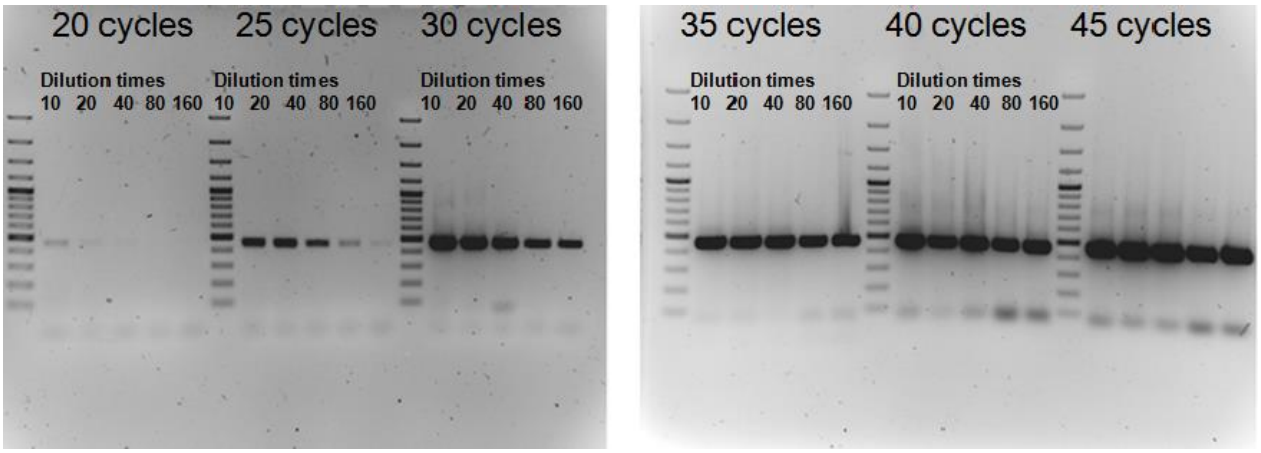


Figure 4.
RT-PCR results of Rh-β-actin performed with different number of cycles and serial dilutions of cDNA.

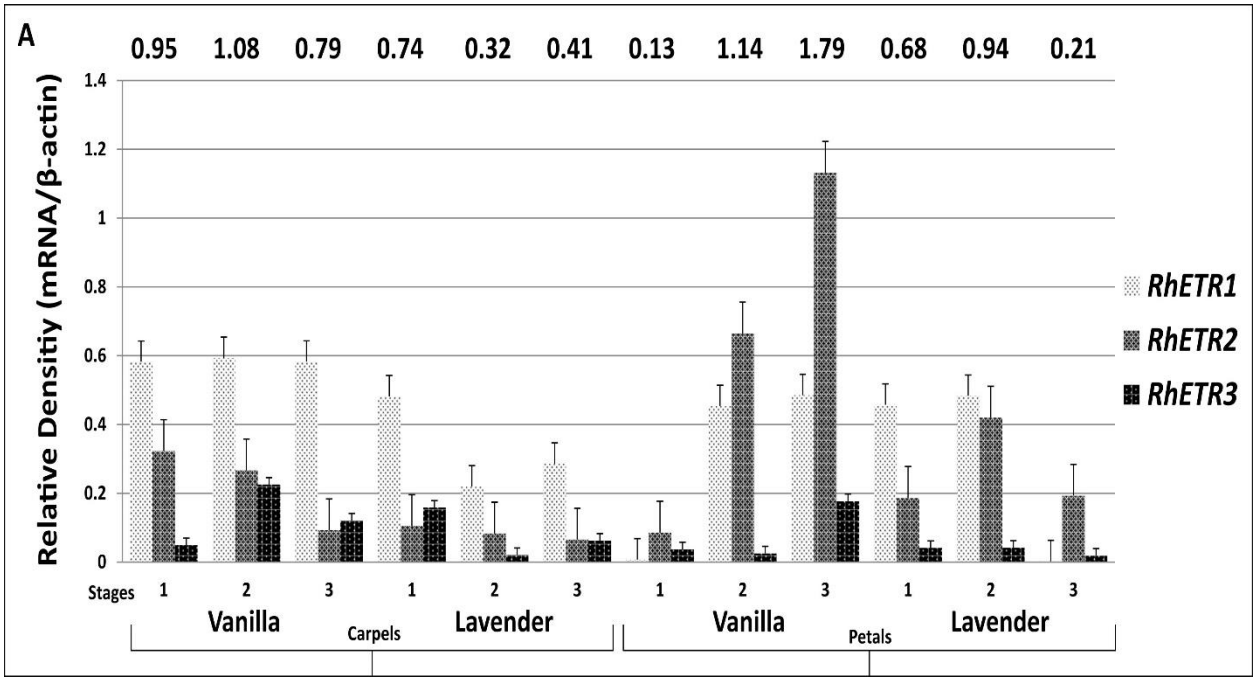
3.3.2 Optimization of RT-PCR of *Rh-β-actin* by Modification of cDNA Concentration

Depending on the fact that housekeeping genes should expressed similarly between different tissues, cDNA concentration was optimized depend on transcript levels obtained from β-action, by increasing or decreasing the cDNA concentration from different tissues, trying to get similar transcript levels, and then use it as control to compare the transcript level of other genes studied. Corrections of amplicon length have been made depending on the β-actin amplicon size, so that the corrections factor equals to 395 divided by length of amplicon of each gene. Then each expression value (and SEM) were multiplied by this correction factor to correct for different lengths of β-actin amplicon and amplicon of analyzed gene (and to make it possible to compare e.g. RhETR1 and RhETR2 in the same tissue). In order to compare the expression of each genes, we calculated also the cumulative expressions for receptors, ACSs, CTRs and EIN3/EIL, and wrote it above each column (expression value), this made it easier to overlook and analyze the total expression of proteins with similar or identical function.

3.3.3 Expression analysis by RT-PCR

3.3.3.1 Ethylene receptors (*RhETR1-4*)

RhETR1, RhETR2 and RhETR3 transcripts were detected in all tissues tested for both 'Vanilla' and 'Lavender' (Fig. 5A). Expression of RhETR4 was not detected, regardless of the type of tissue or growth stage (data not shown). In carpels, the amount of RhETR1 mRNA in 'Vanilla' was higher than for 'Lavender' at all developmental stages (Fig. 5A). In petals, the amount of RhETR2 mRNA was higher for 'Vanilla' than for 'Lavender' at stage 2 and 3 and all three transcripts reached their maximum at stage 3 for 'Vanilla' but stage 2 for 'Lavender'. In floral buds and in leaves the level of all three receptor transcripts was higher for 'Vanilla' than for 'Lavender'. RhETR3 mRNA was higher for 'Vanilla' in buds and leaves whereas it was not expressed in Lavender in the same tissues (Fig. 5B). The total amount of transcript for the three receptors (estimated as the sum of the column heights) was higher for 'Vanilla' than for 'Lavender' in all cases except for stage 1 petals.



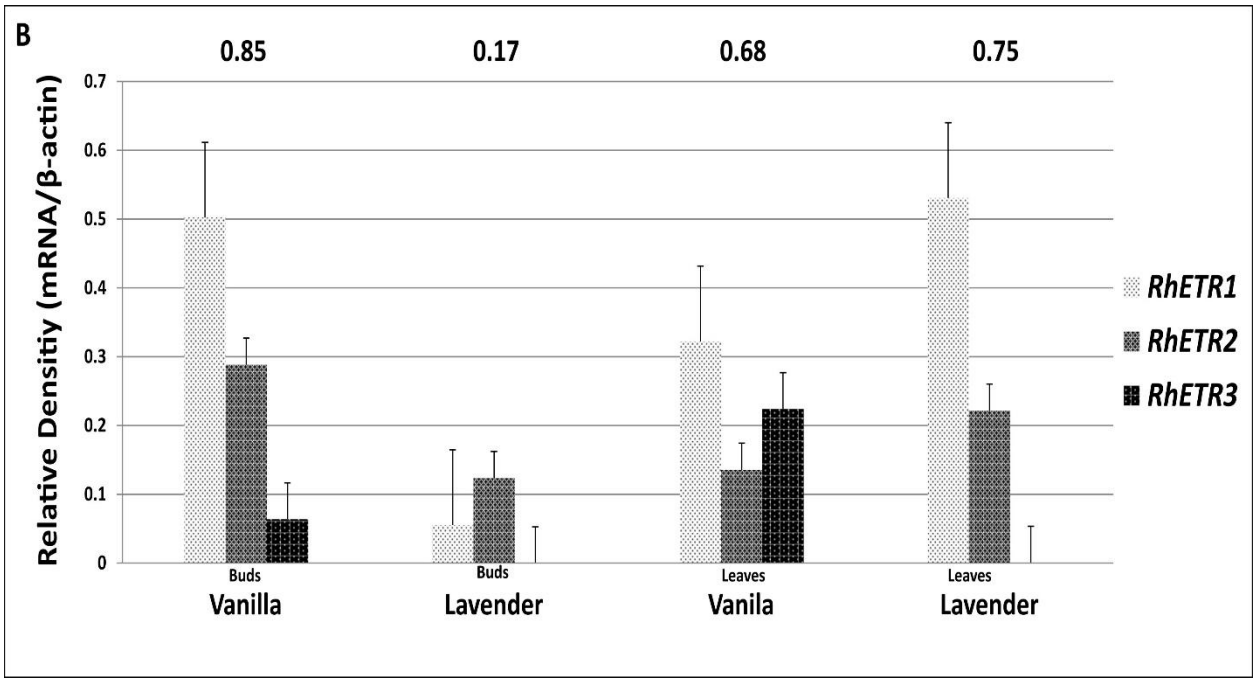


Figure 5.
Expression patterns for RhETR1, RhETR2 and RhETR3 (A) at three developmental stages of carpels and petals, and (B) in floral buds and leaves of 'Vanilla' and 'Lavender' as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard Rhβ-actin, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions, error bars are standard error of the mean. The numbers at the top are the sum of the heights of the columns for RhETR1-3.

For the selected F1 genotypes (Table 1) expression of RhETR1-4 was measured in petals of fully blooming flowers (stage 2, Fig. 2), floral buds and leaves from each of the selected F1 genotypes. As for the parents, no expression of RhETR4 was detected in the investigated tissues. In all cases except for genotype 48 buds the dominant transcripts are RhETR2 (Fig. 6). In general, the differences between the genotypes of low and high ethylene sensitivity are small, and less conspicuous than some of the differences seen between 'Vanilla' and 'Lavender'.

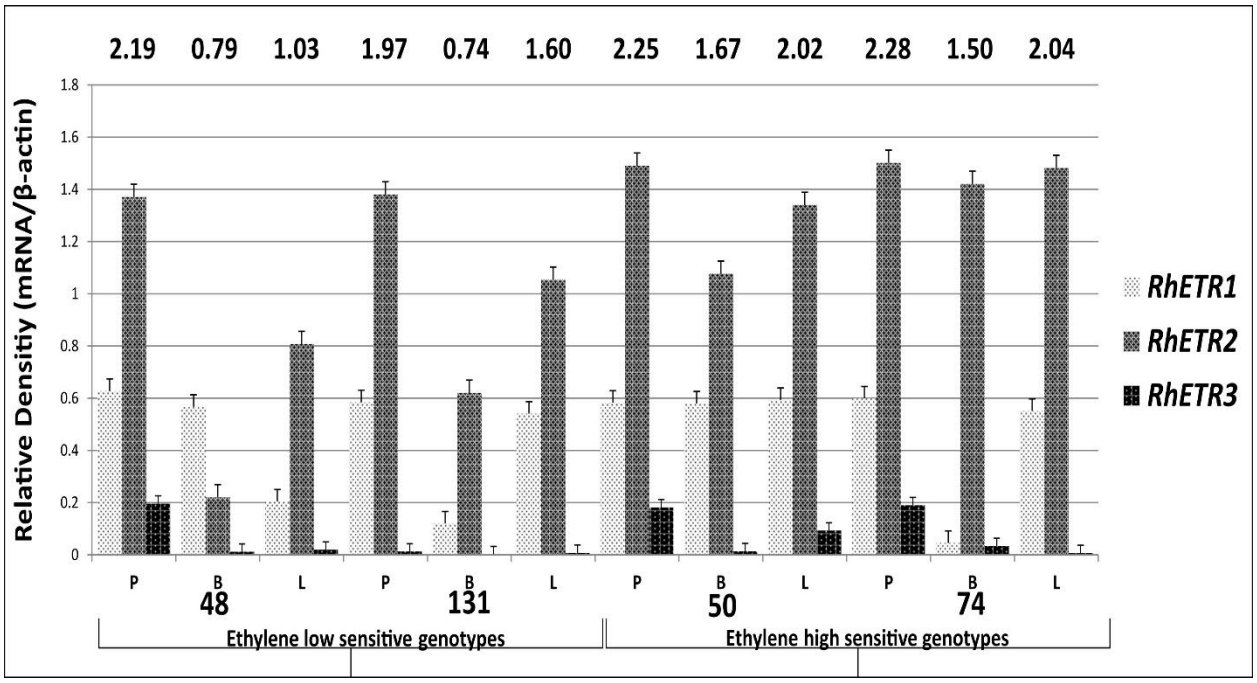


Figure 6.
Expression patterns for RhETR1, RhETR2 and RhETR3 of F1 plants in petals (P), floral buds (B) and leaves (L) as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard Rhβ-actin, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions, error bars are standard error of the mean. The numbers at the top are the sum of the heights of the columns for RhETR1-3.

3.3.3.2 Ethylene biosynthesis enzymes (RhACS1-5)

Expression of the ethylene biosynthetic enzyme genes RhACS1-5 was analyzed in 'Vanilla' and 'Lavender'. Whereas RhACS1 and RhACS2 transcripts were detected in all investigated tissues for both cultivars (Fig. 7), RhACS3-5 transcripts were not detected in any of the investigated tissues (data not shown). In 'Vanilla' and 'Lavender' carpels and in 'Vanilla' petals, buds and leaves RhACS2 was more expressed than RhACS1, whereas the opposite was the case for 'Lavender' petals (except for stage 1), buds and leaves. The sum of the two transcript levels is in all cases larger for 'Vanilla' than for 'Lavender'.

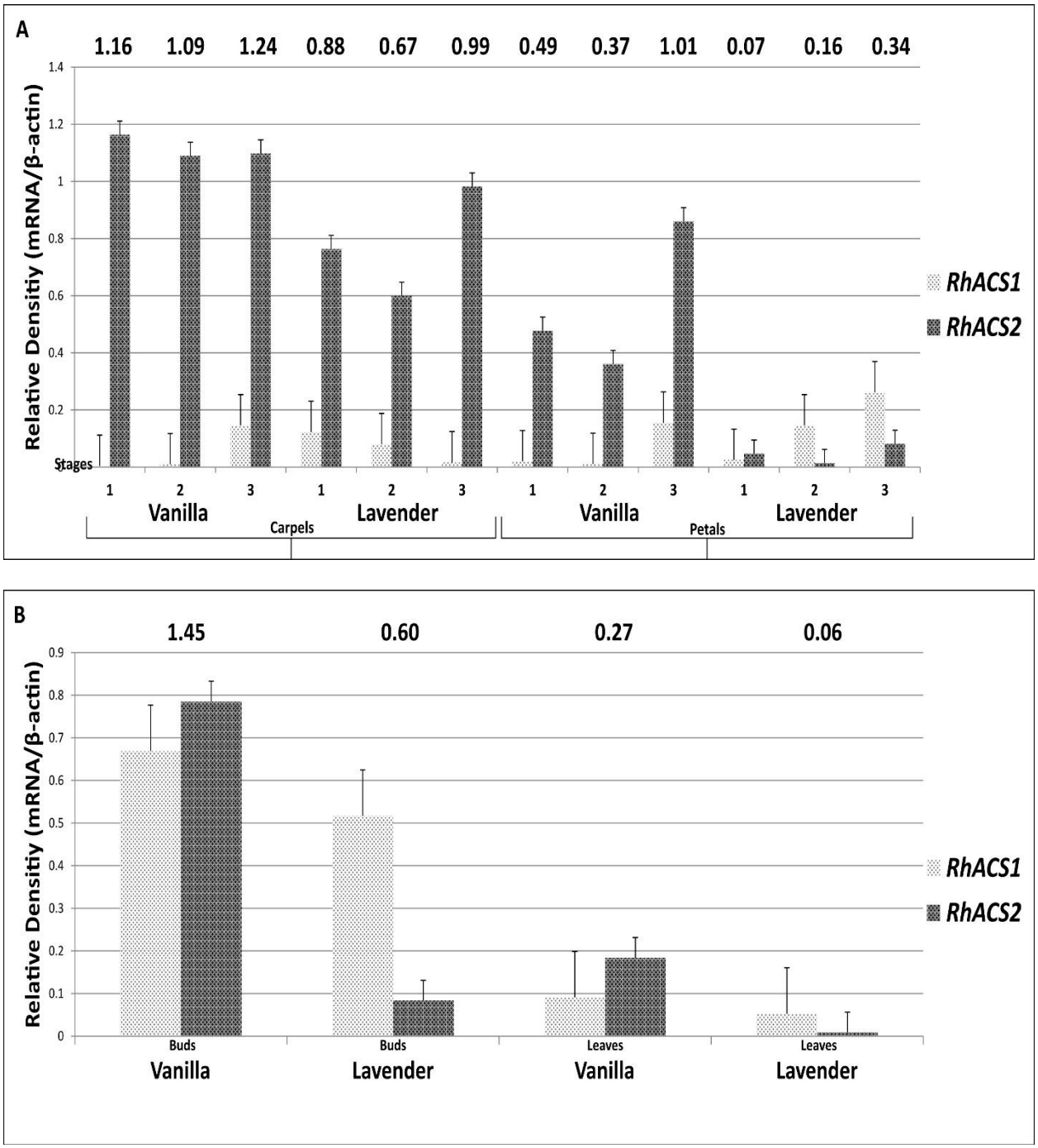


Figure 7.
Expression patterns for RhACS1 and RhACS2 (A) at three developmental stages of carpels and petals, and (B) in floral buds and leaves of 'Vanilla' and 'Lavender' as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard Rhβ-actin, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions, error bars are standard error of the mean. The numbers at the top are the sum of the heights of the columns for RhACS1 and RhACS2.

As for the parent plants, expression analysis of stage 2 petals, buds and leaves from the selected F1 plants detected RhACS1 and RhACS2 transcripts (Fig. 8), but no RhACS3, RhACS4 and RhACS5 transcripts (data not shown), in the investigated tissues. The expression of RhACS1 was always higher than the RhACS2 expression in petal tissue, as for 'Lavender' but not for 'Vanilla' (Fig. 7A), regardless of sensitivity to ethylene (Fig. 8). In addition, the sum of the two transcript levels in petals is larger than for both 'Lavender' and 'Vanilla' for all 4 genotypes. For leaves and buds the patterns were more variable, but without conspicuous correlation with ethylene sensitivity.

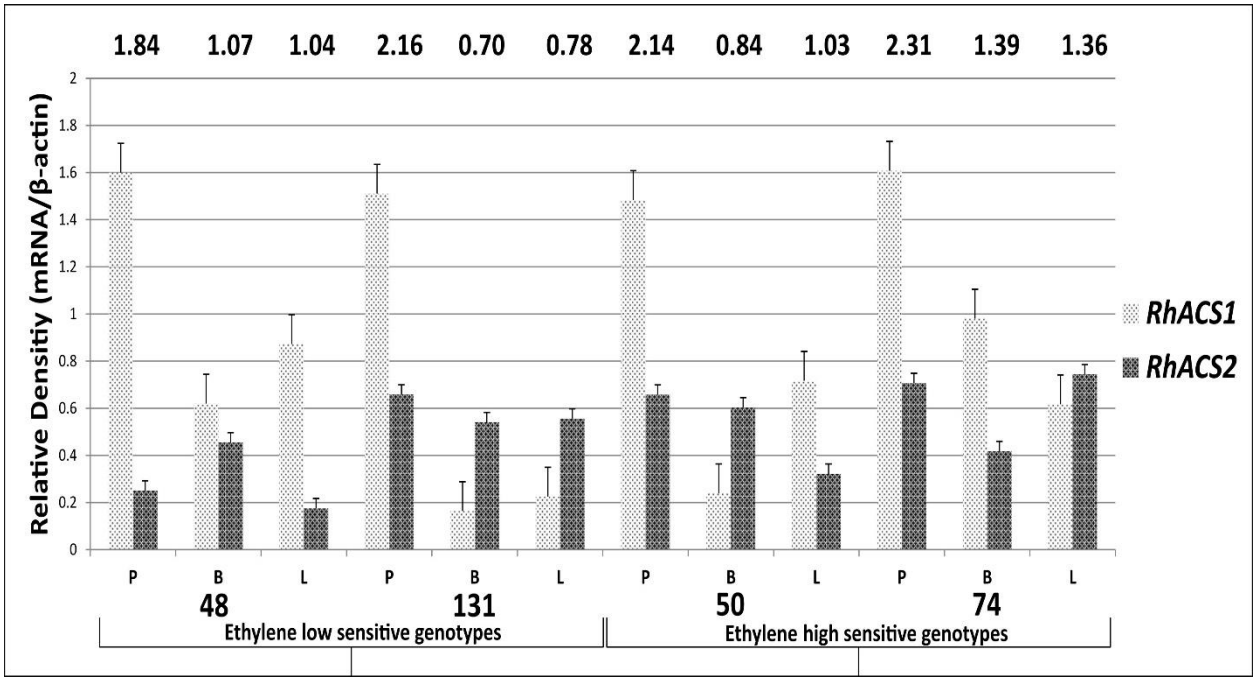
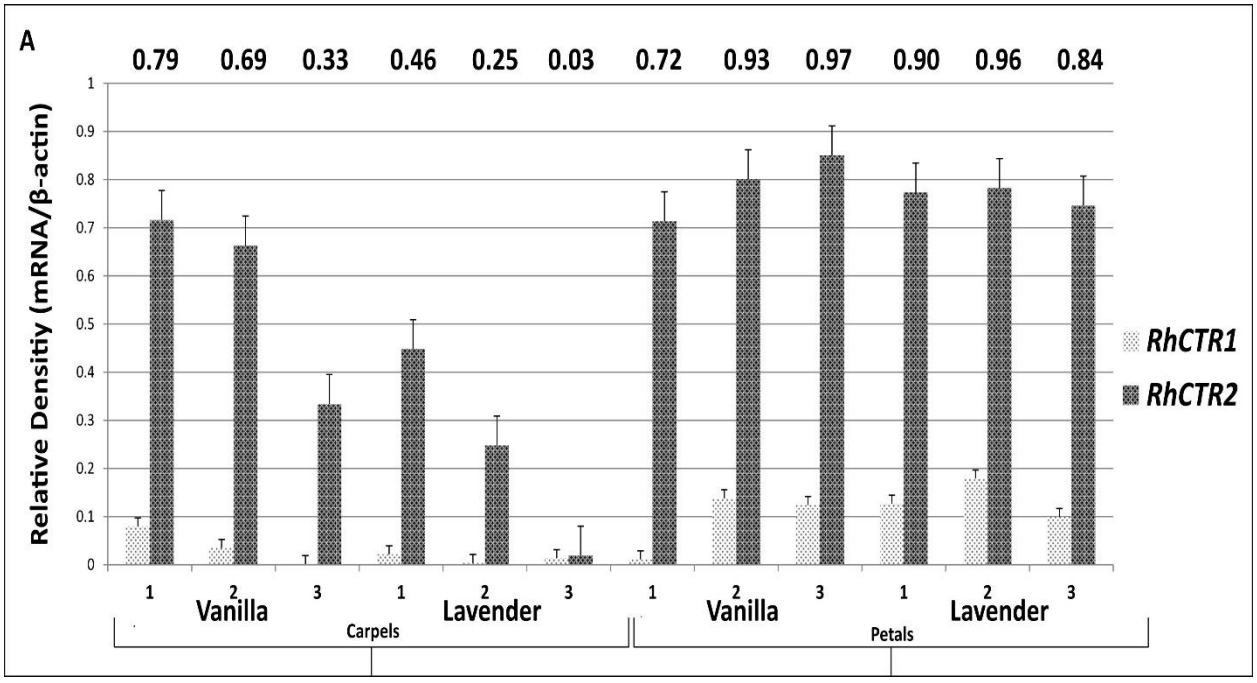


Figure 8.
Expression patterns for RhACS1 and RhACS2 of F1 plants in petals (P), floral buds (B) and leaves (L) as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard Rhβ-actin, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions, error bars are standard error of the mean. The numbers at the top are the sum of the heights of the columns for RhACS1 and RhACS2.

3.3.3.3 Ethylene signal transduction (RhCTR1 and RhCTR2)

Expression of the RhCTR1 and RhCTR2 genes, which encode homologous protein kinases involved in ethylene signal transduction, were analyzed in 'Vanilla' and 'Lavender' (Fig. 9). RhCTR2 was significantly more expressed than RhCTR1 in all investigated tissues, except for 'Lavender' stage 3 carpels where both showed very low expression (Fig. 9A). The total amount of transcript for the two genes (estimated as the sum of the column heights) varied, with the exception just mentioned, from 0.33 (for 'Vanilla' stage 3 carpels) and 0.97 (for 'Vanilla' stage 3 petals). It was relatively constant for all 6 samples of petals.



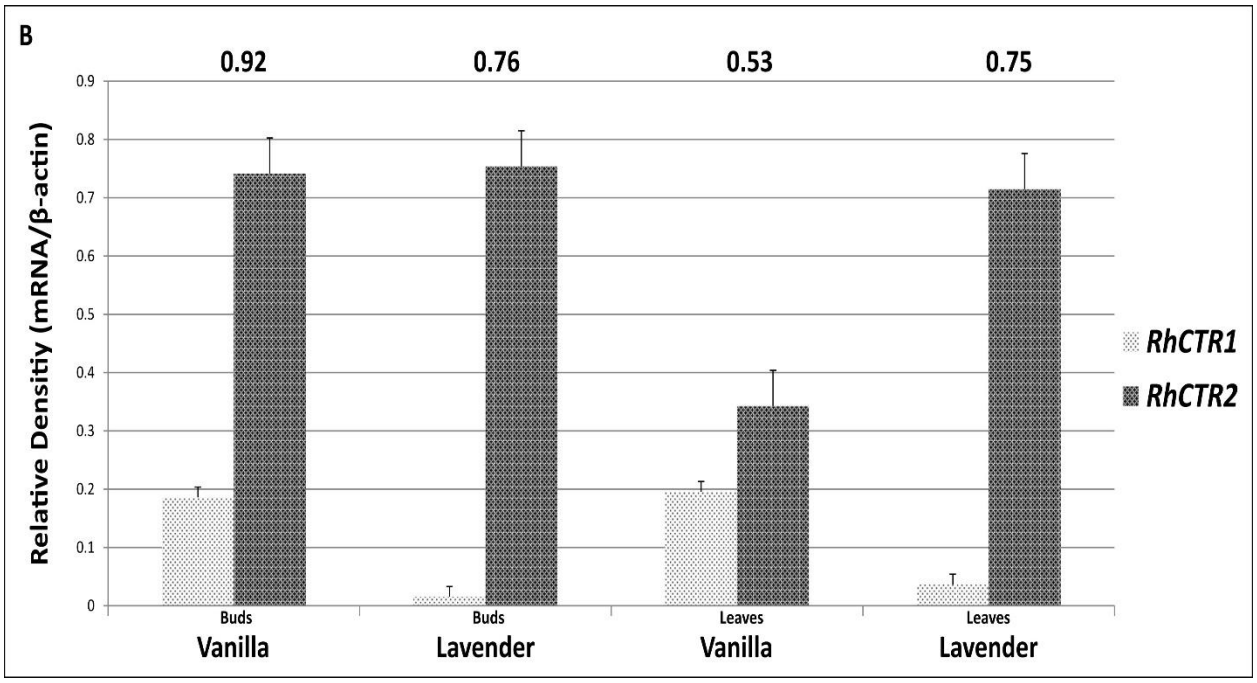


Figure 9.
Expression patterns for RhCTR1 and RhCTR2 (A) at three developmental stages of carpels and petals, and (B) in floral buds and leaves of 'Vanilla' and 'Lavender' as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard Rhβ-actin, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions, error bars are standard error of the mean. The numbers at the top are the sum of the heights of the columns for RhCTR1 and RhCTR2.

For the selected F1 plants the expression of RhCTR1 was significantly higher than, and that of RhCTR2 was, in general, at the same level as, the expressions found in 'Vanilla' and 'Lavender' (Fig. 10). Accordingly, the sum of the expressions was higher than for the parent plants.

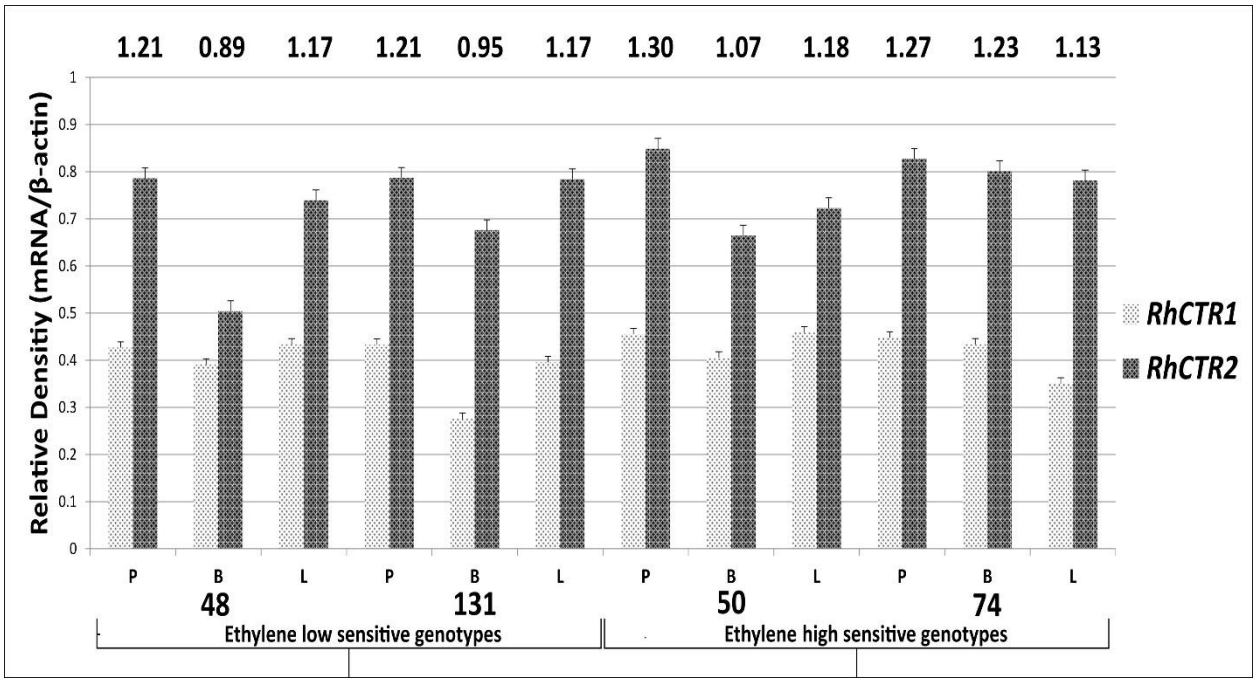


Figure 10.
Expression patterns for RhCTR1, RhCTR2 of F1 plants in petals (P), floral buds (B) and leaves (L) as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard Rhβ-actin, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions, error bars are standard error of the mean. The numbers at the top are the sum of the heights of the columns for RhCTR1 and RhCTR2.

3.3.3.4 Ethylene transcription factors (RhEIN3 and RhEIL)

Expression of the RhEIN3 and RhEIL genes, which encode homologous transcription factors involved in the ethylene signal pathway, were detected in all investigated tissues from 'Vanilla' and 'Lavender' (Fig. 11). In all cases, except for 'Vanilla' stage 1 carpels and 'Lavender' stage 3 petals, buds and leaves, the expression of RhEIL exceeded that of RhEIN3. In petals, the amount of both transcripts was maximal at stage 2 and 3 for 'Vanilla' but at stage 1 and 2 for 'Lavender' (Fig. 11A). As for the parent plants, expression analysis of stage 2 petals, buds and leaves from the selected F1 plants detected both transcripts (Fig. 12) and more RhEIL than RhEIN3 transcript. There is only little variation between expression in the 4 genotypes. For the petals, the total

expressions are similar to that of stage 2 petals from the parent plants, and for leaves the total expressions are also similar.

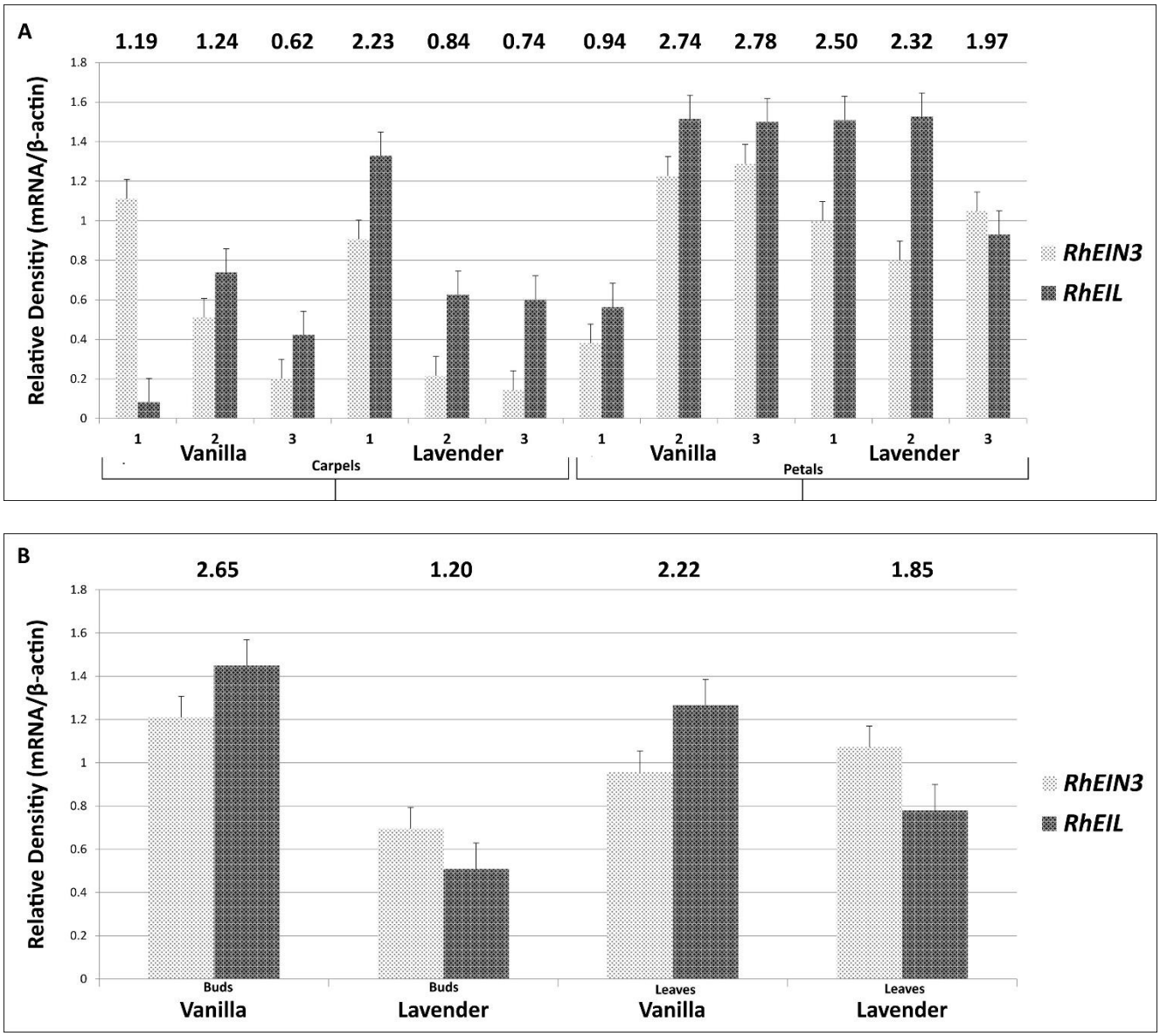


Figure 11.
Expression patterns for RhEIN3 and RhEIL (A) at three developmental stages of carpels and petals, and (B) in floral buds and leaves of 'Vanilla' and 'Lavender' as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard Rhβ-actin, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions, error bars are standard error of the mean. The numbers at the top are the sum of the heights of the columns for RhEIN3 and RhEIL.

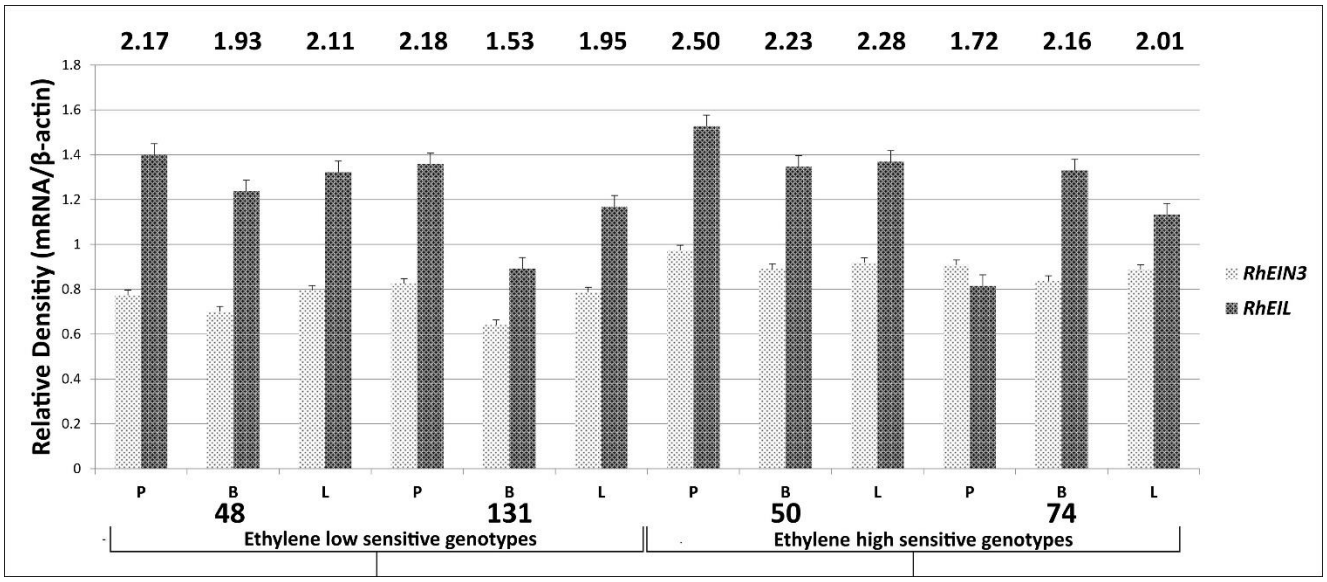


Figure 12.
Expression patterns for RhEIN3 and RhEIL of F1 plants in petals (P), floral buds (B) and leaves (L) as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard Rhβ-actin, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions, error bars are standard error of the mean. The numbers at the top are the sum of the heights of the columns for RhEIN3 and RhEIL.

3.3.4 Expression analysis by Reverse Northern dot blot hybridization

3.3.4.1 Between 'Vanilla' and 'Lavender' in different tissues

a) Ethylene receptors (RhETR1-4)

The expression of *RhETRs* in 'Vanilla' was higher than in 'Lavender' as seen from (Fig. 13) in Petals (A) and in whole floral buds (B). The differences in expression between 'Vanilla' and 'Lavender' were easy to perceive in both tissues. In both, petals and floral buds, the expression of *RhETR3* was the greatest compared to *RhETR1*, *RhETR3*, and *RhETR4*. In all treatments, as expected, the hybridization of DNA to the probe became higher high as the concentration if DNA on filter increased.

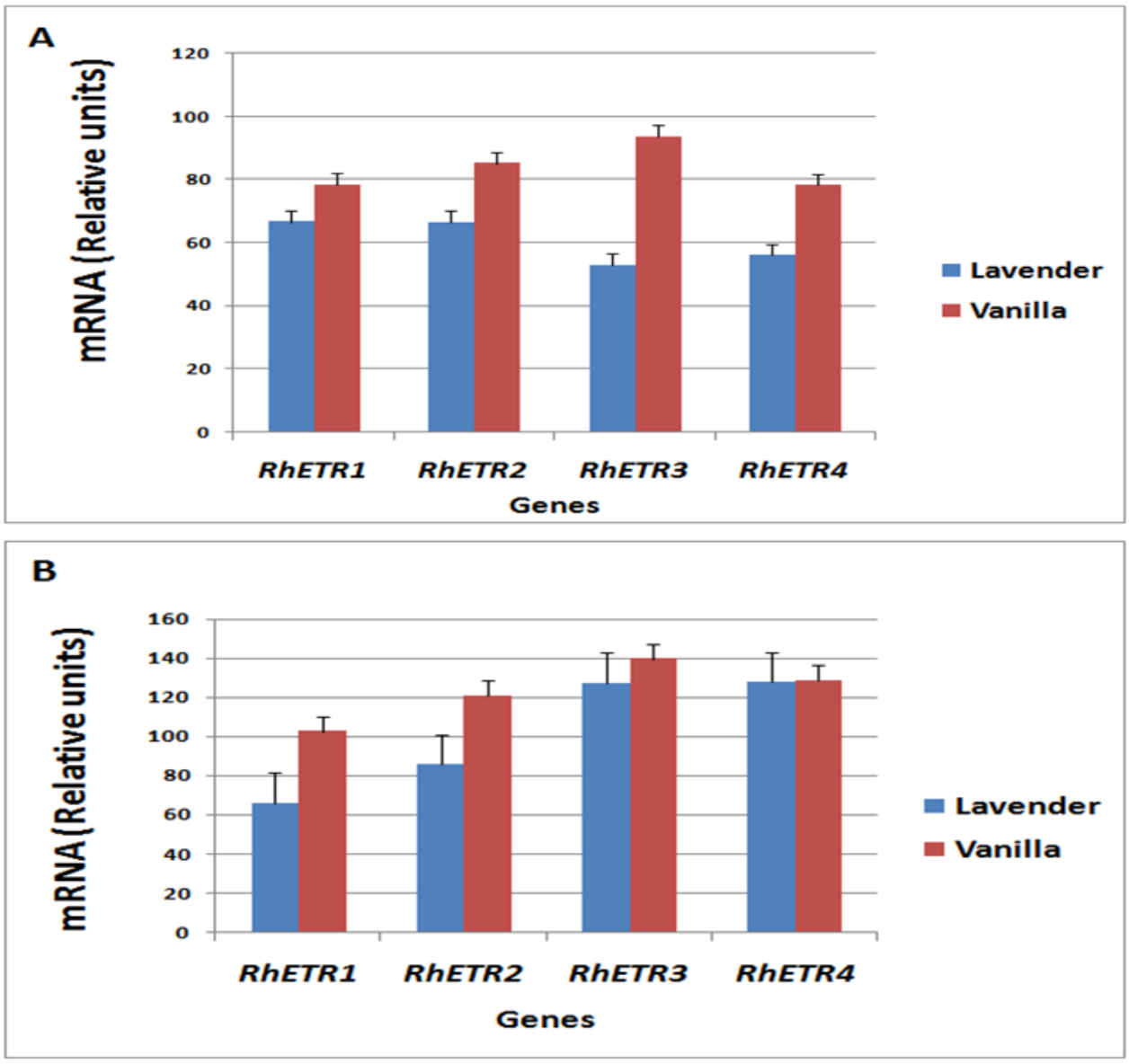


Figure 13.

Reverse Northern dot blots of RhETR1-4. Equal volumes of various amount of DNA (0.5, 0.1, or 0.02 µg) of the amplified genes fragments, together with various amount of Rh-β-actin DNA (0.5, 0.1, or 0.02 µg) were blotted on Hybond-N+ nylon transfer membrane using the micropipette. After denaturation, neutralization and DNA fixation, the filters containing the spotted DNA were hybridized to Dig-labelled cDNA probes from specified tissues. (A; petals, B; whole floral buds) Histogram summarizing densitometric analysis of Reverse Northern blots normalized to Rhβ-actin. Values represent means ± standard error of the mean (S.E.M.) or all genes investigated, the right cDNA size was amplified (see Table 2). Densitometric analysis was performed using ImageQuant TL (Amersham, GE Healthcare UK limited, Buckinghamshire, UK).

b) Ethylene biosynthesis enzymes genes (RhACS1-5)

Reverse Northern dot blots showed that the expression of *RhACSs* was high in petals and whole floral buds. In petals of 'Vanilla', the expression of *RhACS1-5* was higher than in 'Lavender' as seen from (Fig. 14A, B). The same results were also obtained in whole floral bud except that in *RhACS3-5*, in which the expression was higher in 'Lavender' than in 'Vanilla'. There was an interesting trend in both tissues studied in which the expression of *RhACS2* had consistently higher expression levels compared with other *RhACSs*, in addition to, the expression of *RhACS2* was higher in 'Vanilla' than in 'Lavender' in both tissues investigated.

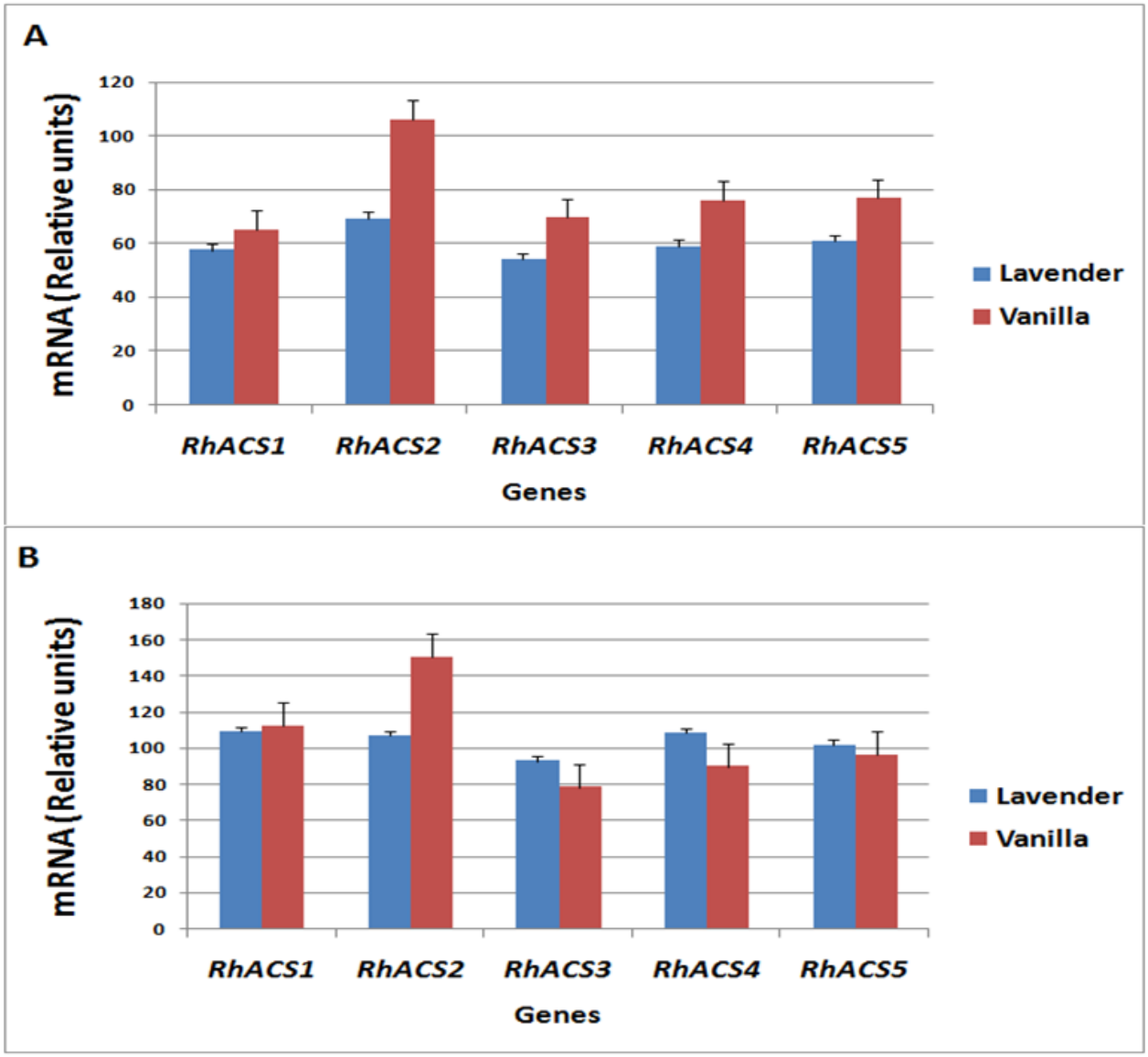


Figure 14.
Reverse Northern dot blots of RhACS1-5. Equal volumes of various amount of DNA (0.5, 0.1, or 0.02 µg) of the amplified genes fragments, together with various amount of Rh-β-actin DNA (0.5, 0.1, or 0.02 µg) were blotted on Hybond-N+ nylon transfer membrane using the micropipette. After denaturation, neutralization and DNA fixation, the filters containing the spotted DNA were hybridized to Dig-labelled cDNA probes from specified tissues. (A; petals, B; whole floral buds) Histogram summarizing densitometric analysis of Reverse Northern blots normalized to Rhβ-actin. Values represent means ± standard error of the mean (S.E.M.) or all genes investigated, the right cDNA size was amplified (see Table 2). Densitometric analysis was performed using ImageQuant TL (Amersham, GE Healthcare UK limited, Buckinghamshire, UK).

c) Ethylene signal transduction (RhCTR1-2) and transcription factors (RhEIN3, RhEIL)

The expression levels of *RhCTR1-2*, *RhEIN3*, and *RhEIL*, were higher in Vanilla than in lavender in both petals and whole floral buds (Fig. 15A, B). As a general trend, in both tissues, RhCTR1

expression was the highest, and then RhEIN3 followed by RhEIL and the lowest expression was RhCTR2.

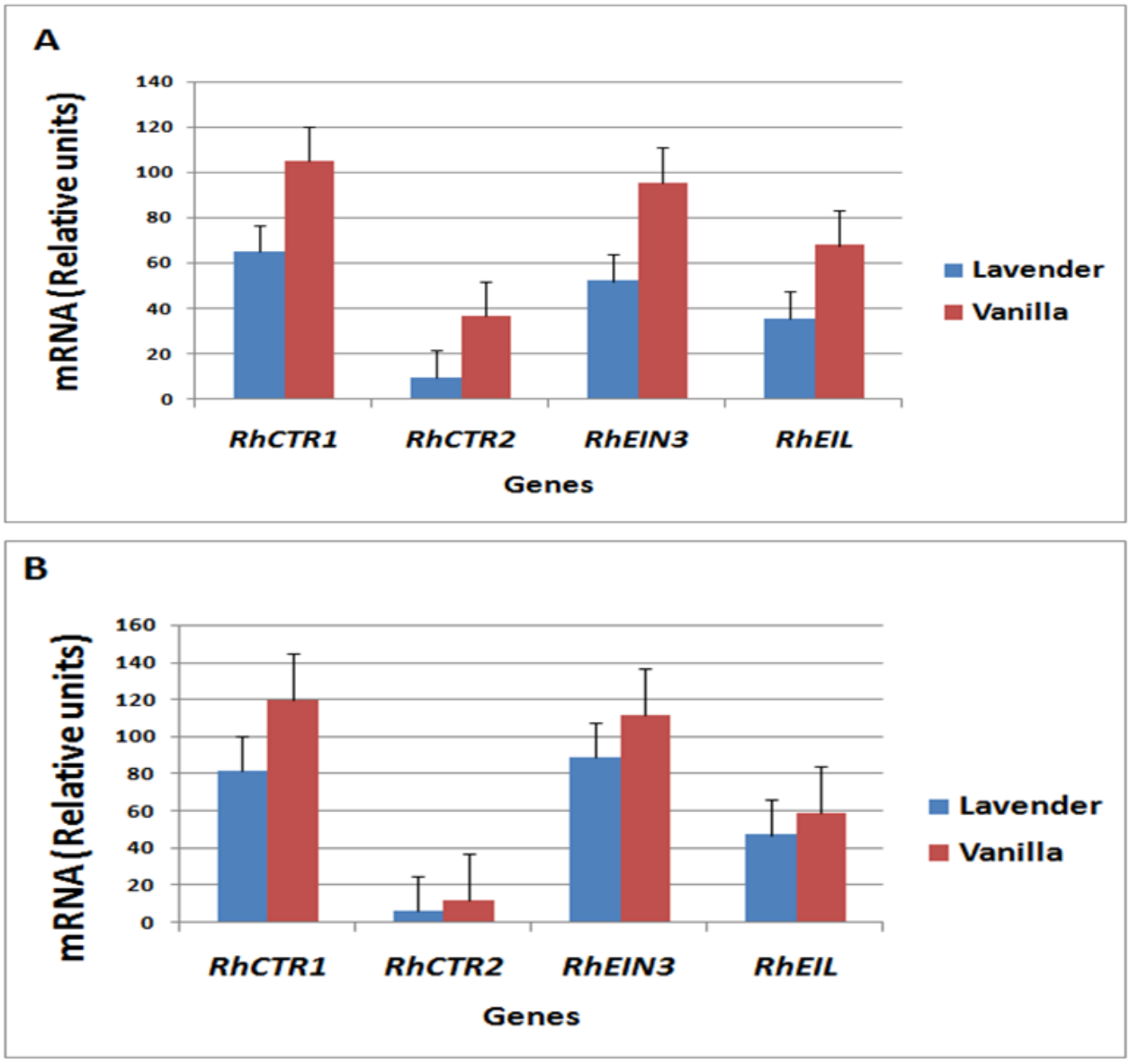


Figure 15.

Reverse Northern dot blots of RhCTR1-2, RhEIN3 and RhEIL. Equal volumes of various amount of DNA (0.5, 0.1, or 0.02 µg) of the amplified genes fragments, together with various amount of Rh-β-actin DNA (0.5, 0.1, or 0.02 µg) were blotted on Hybond-N+ nylon transfer membrane using the micropipette. After denaturation, neutralization and DNA fixation, the filters containing the spotted DNA were hybridized to Dig-labelled cDNA probes from specified tissues. (A; petals, B; whole floral buds) Histogram summarizing densitometric analysis of Reverse Northern blots normalized to Rhβ-actin. Values represent means ± standard error of the mean (S.E.M.) or all genes investigated, the right cDNA size was amplified (see Table 2). Densitometric analysis was performed using ImageQuant TL (Amersham, GE Healthcare UK limited, Buckinghamshire, UK).

3.3.4.2 Between F1 plants

The mRNA expression patterns of specific genes (*RhETRs*, *RhACSs*, *RhCTRs*, *RhEIN3* and *RhEIL*) in total RNA from 'Vanilla' X 'Lavender' (F1 plants), 'Lavender' X 'Lavender' ('Lavender' selfing), and lavender X lavender with vanilla X vanilla (LXL with VXV) was studied by reverse Northern dot blot (Fig. 16-20). The tissues studied were collected from petals in the full blooming stage of the flower. The expression levels were sorted according to gene group.

a) Ethylene receptors (RhETR1-4)

In F1 plants, different expression patterns of *RhETR1-4* were detected in two selected plants one is highly sensitive to ethylene (No. 74) and the other is lowly sensitive to ethylene (No. 48). The expression of both *RhETR1* and *RhETR3* was higher in ethylene high sensitive plant than in ethylene low sensitive plant, conversely, the expression of *RhETR4* was higher in ethylene low sensitive plants. The expression of *RhETR2* in both plants showed no difference (Fig.16A, B).

In lavender selfing, expression of *RhETR1* and RhETR2 was higher in ethylene high sensitive plant than in ethylene low sensitive plant. The expression level of RhETR3 was almost the same in both plants, while the expression of RhETR4 was higher in ethylene low sensitive plant than in ethylene low sensitive plant (Fig.16 C, D). when comparing vanilla selfing plants with Lavender selfing plants, the expression of All *RhETRs* studied was higher in (V x V) plant than in (L X L) plant, the expression of ethylene receptor studied increased gradually from *RhETR1* to RhETR4 in which high expressions were detected.

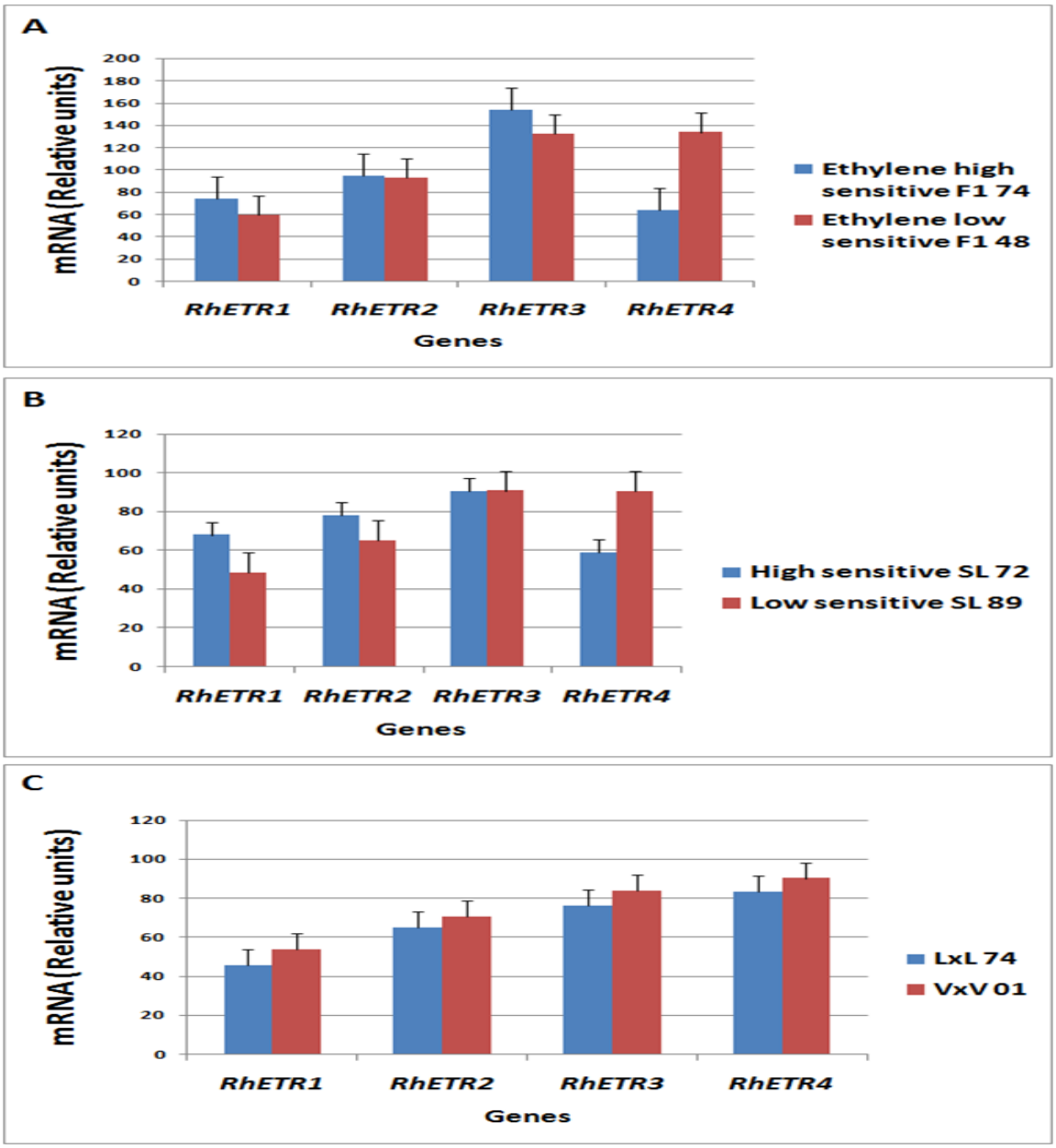


Figure 16.

Reverse Northern dot blots. Equal volumes of various amount of DNA (0.5, 0.1, or 0.02 µg) of the amplified genes fragments, together with various amount of Rh-β-actin DNA (0.5, 0.1, or 0.02 µg) were blotted on Hybond-N+ nylon transfer membrane using the micropipette. After denaturation, neutralization and DNA fixation, the filters containing the spotted DNA were hybridized to Dig-labelled cDNA probes from specified tissues. (A; petals, C; whole floral buds) Autoradiogram obtained after Reverse Northern blot hybridization of the spotted cloned gene fragments to a probe prepared by reverse transcription of 25 µg of total RNA purified from the full opening flower petals from both vanilla and lavender. (B; Petals, D; whole floral buds) Histogram summarizing densitometric analysis of Reverse Northern blots as described in (A) normalized to Rhβ-actin. Values represent means ± standard error of the mean (S.E.M.) or all genes investigated, the right cDNA size was amplified (see Table 2). Densitometric analysis was performed using ImageQuant TL (Amersham, GE Healthcare UK limited, Buckinghamshire, UK).

b) Ethylene biosynthesis enzymes genes (RhACS1-5)

In F1 plants, lavender selfing plants, and LXL with VXV plants, reverse Northern blot analysis showed that RhACS2 transcript was excessively detected (Fig. 17A-F). In F1 plants, a hybridization signal of RhACS1 was higher in ethylene low sensitive plant than in ethylene high sensitive plant. While there were no differences in lavender selfing plants and LXL with VXV plants. Transcripts of RhACS3-5 showed no differences between both plant varieties (Fig. 17A-F).

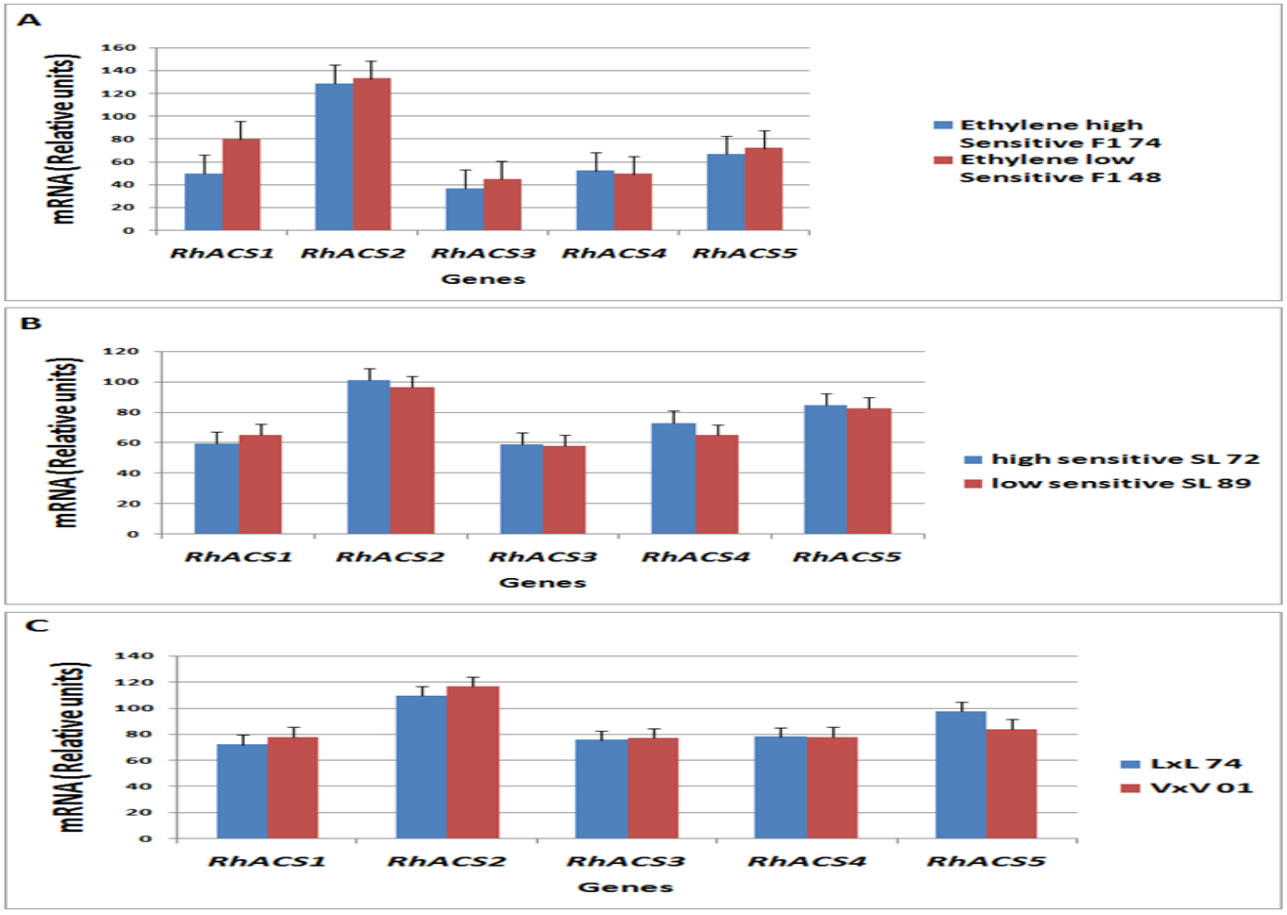


Figure 17.
Reverse Northern dot blots. Equal volumes of various amount of DNA (0.5, 0.1, or 0.02 µg) of the amplified genes fragments, together with various amount of Rh-β-actin DNA (0.5, 0.1, or 0.02 µg) were blotted on Hybond-N+ nylon transfer membrane using the micropipette. After denaturation, neutralization and DNA fixation, the filters containing the spotted DNA were hybridized to Dig-labelled cDNA probes from specified tissues. (A; petals, C; whole floral buds) Autoradiogram obtained after Reverse Northern blot hybridization of the spotted cloned gene fragments to a probe prepared by reverse transcription of 25 µg of total RNA purified from the full opening flower petals from both vanilla and lavender. (B; Petals, D; whole floral buds) Histogram summarizing densitometric analysis of Reverse Northern blots as described in (A) normalized to Rhβ-actin. Values represent means ± standard error of the mean (S.E.M.) or all genes investigated, the right cDNA size was amplified (see Table 2). Densitometric analysis was performed using ImageQuant TL (Amersham, GE Healthcare UK limited, Buckinghamshire, UK).

c) Ethylene signal transduction (RhCTR1-2) and transcription factors (RhEIN3, RhEIL)

The expression of ethylene signal transduction and transcription factors In F1 plants, lavender selfing plants, and LXL with VXV plants, clearly followed the same patterns, since the hybridization signals from the reverse Northern plot appeared without any differences between each group of investigated plants except in F1 plants, in which RhCTR1 was highly expressed in ethylene low sensitive plants than in ethylene high sensitive plant (Fig. 18A-C). The Hybridization signal of RhCTR2 was very weak, and this probably due to the degradation or washing of DNA on the membrane since it has been reused many times.

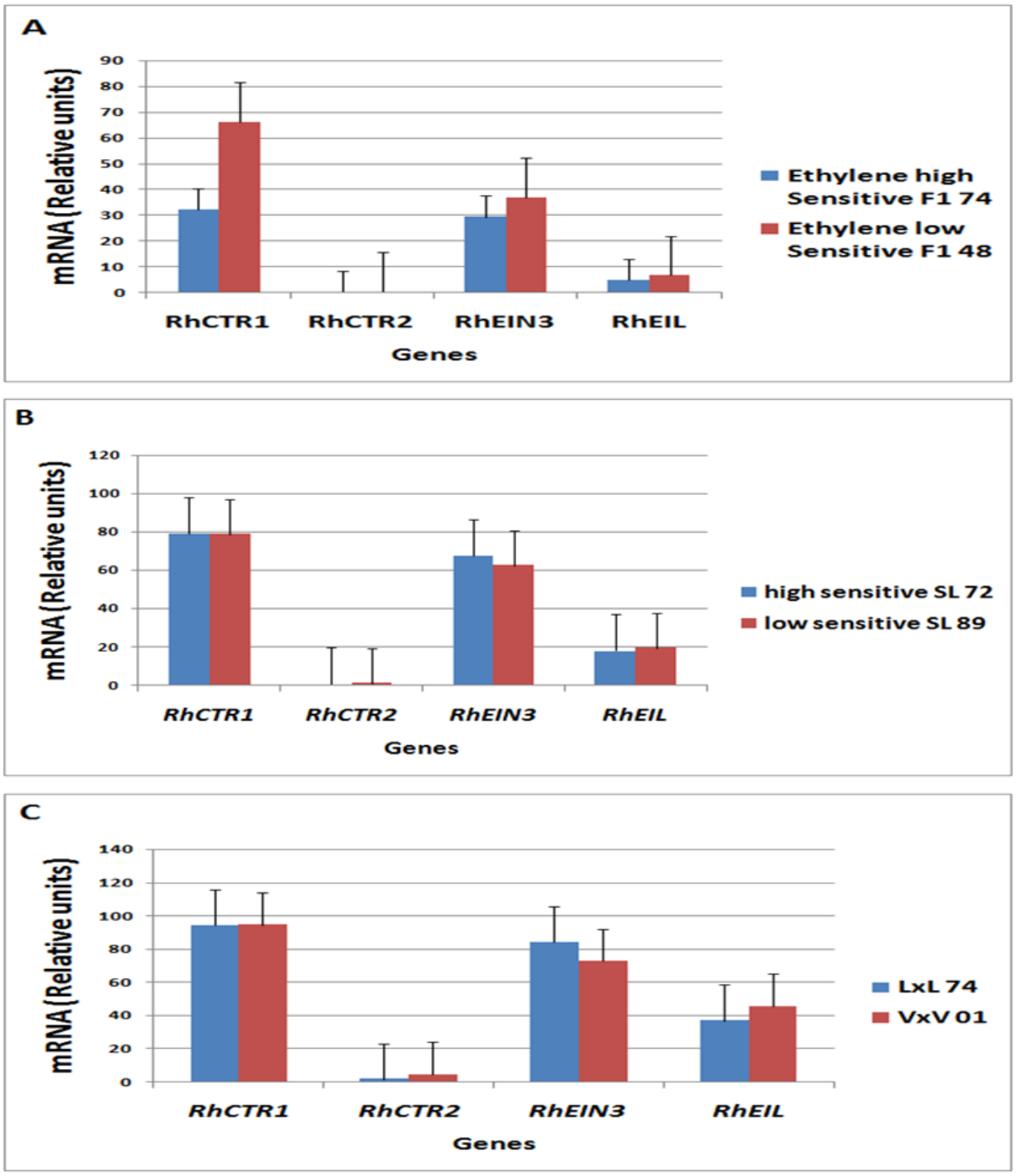


Figure 18.

Reverse Northern dot blots. Equal volumes of various amount of DNA (0.5, 0.1, or 0.02 µg) of the amplified genes fragments, together with various amount of Rh-β-actin DNA (0.5, 0.1, or 0.02 µg) were blotted on Hybond-N+ nylon transfer membrane using the micropipette. After denaturation, neutralization and DNA fixation, the filters containing the spotted DNA were hybridized to Dig-labelled cDNA probes from specified tissues. (A; petals, C; whole floral buds) Autoradiogram obtained after Reverse Northern blot hybridization of the spotted cloned gene fragments to a probe prepared by reverse transcription of 25 µg of total RNA purified from the full opening flower petals from both vanilla and lavender. (B; Petals, D; whole floral buds) Histogram summarizing densitometric analysis of Reverse Northern blots as described in (A) normalized to Rhβ-actin. Values represent means ± standard error of the mean (S.E.M.) or all genes investigated, the right cDNA size was amplified (see Table 2). Densitometric analysis was performed using ImageQuant TL (Amersham, GE Healthcare UK limited, Buckinghamshire, UK).

3.3.4.3 between ethylene treated or non-treated plants

a) Ethylene receptors (RhETR1-4)

Hybridization signals of ethylene receptors (*RhETR1*, RhETR2 and RhETR4) in floral bud abscission zone of both vanilla and lavender exhibited little differences between ethylene treated and non-treated plants the same expression patterns as seen from the reverse Northern Plot (Fig. 19 A-B) whether they were treated with ethylene or not. In contrast, the expression of ethylene receptor (RhETR3) in 'Vanilla' was higher in the ethylene treated plants than in ethylene non-treated plants, while it was lower in 'Lavender' plants that treated with ethylene.

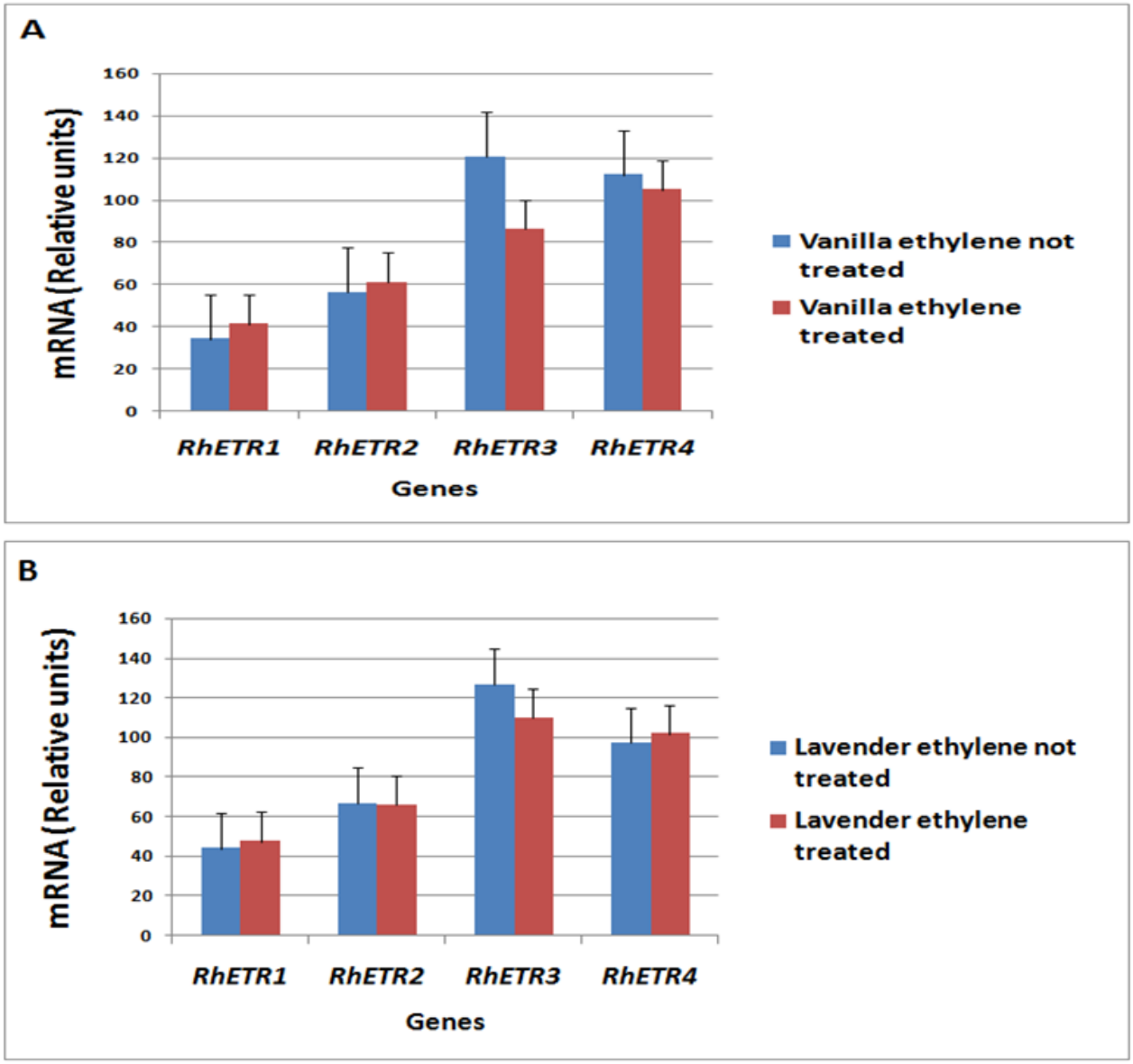


Figure 19.
Reverse Northern dot blots. Equal volumes of various amount of DNA (0.5, 0.1, or 0.02 µg) of the amplified genes fragments, together with various amount of Rh-β-actin DNA (0.5, 0.1, or 0.02 µg) were blotted on Hybond-N+ nylon transfer membrane using the micropipette. After denaturation, neutralization and DNA fixation, the filters containing the spotted DNA were hybridized to Dig-labelled cDNA probes from specified tissues. (A; petals, C; whole floral buds) Autoradiogram obtained after Reverse Northern blot hybridization of the spotted cloned gene fragments to a probe prepared by reverse transcription of 25 µg of total RNA purified from the bud's abscission zone from both vanilla and lavender. (B; Petals, D; whole floral buds) Histogram summarizing densitometric analysis of Reverse Northern blots as described in (A) normalized to Rhβ-actin. Values represent means ± standard error of the mean (S.E.M.) or all genes investigated, the right cDNA size was amplified (see Table 2). Densitometric analysis was performed using ImageQuant TL (Amersham, GE Healthcare UK limited, Buckinghamshire, UK).

b) Ethylene biosynthesis enzymes genes (RhACS1-5)

The pattern of RhACS2 expression differed greatly from other ethylene biosynthesis enzyme genes. The level of RhACS2 mRNA in the bud's abscission zone of ethylene non-treated plants was higher than in bud's abscission zone of ethylene treated plants in 'Lavender', while it was lower in 'Vanilla' (Fig. 20 A-B). in 'Lavender', which is high sensitive ethylene plant, RhACS1, RhACS3, RhACS4, and RhACS5 was expressed more in bud's abscission zone of ethylene non-treated plants than in bud's abscission zone of ethylene treated plants. On the other hand, in 'Vanilla', which is low sensitive ethylene plant, RhACS1, RhACS3, RhACS4, and RhACS5 was expressed more in bud's abscission zone of ethylene treated plants than in in bud's abscission zone of ethylene non-treated plants (Fig. 20 A-B).

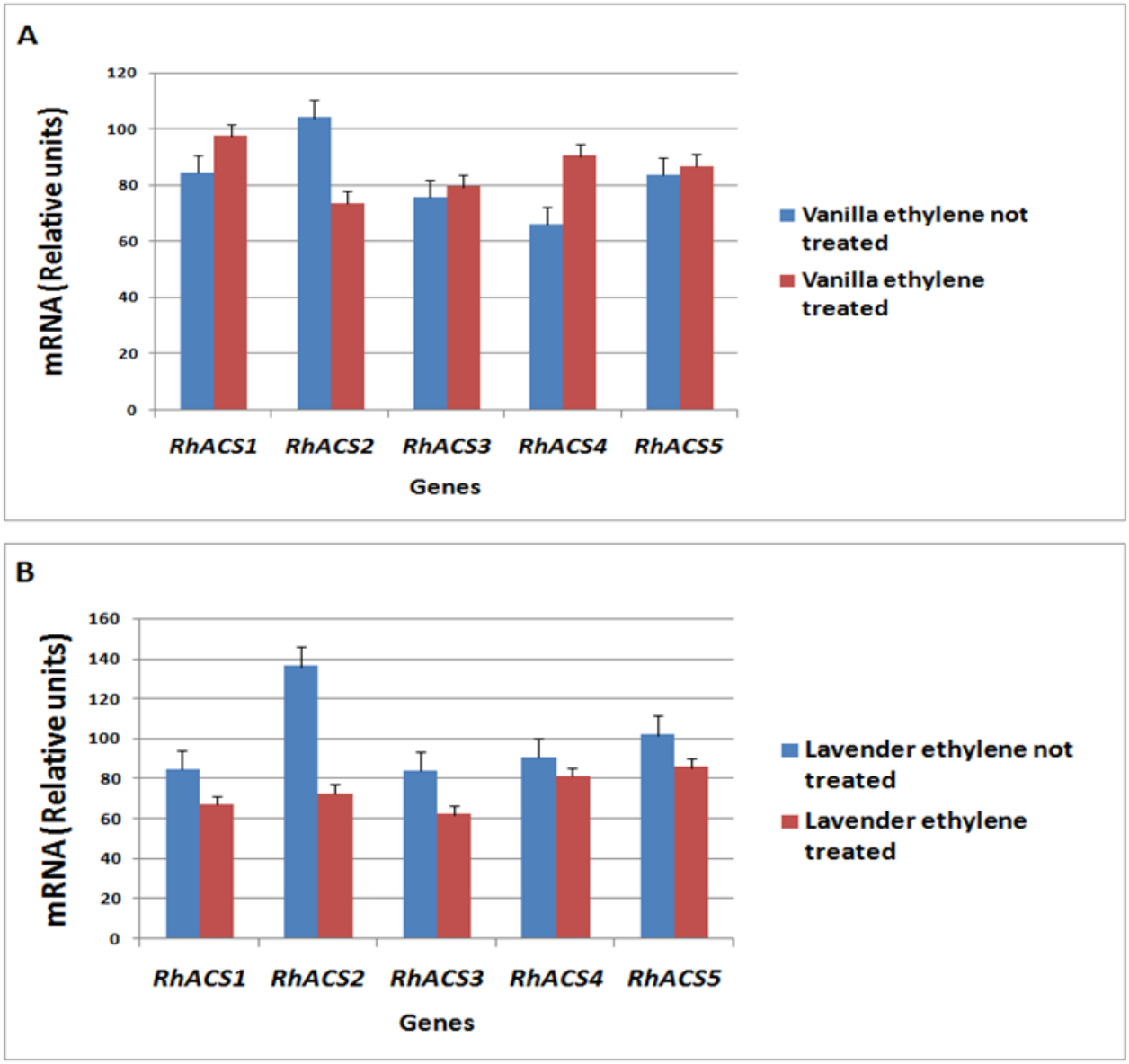


Figure 20.

Reverse Northern dot blots. Equal volumes of various amount of DNA (0.5, 0.1, or 0.02 µg) of the amplified genes fragments, together with various amount of Rh-β-actin DNA (0.5, 0.1, or 0.02 µg) were blotted on Hybond-N+ nylon transfer membrane using the micropipette. After denaturation, neutralization and DNA fixation, the filters containing the spotted DNA were hybridized to Dig-labelled cDNA probes from specified tissues. (A; petals, C; whole floral buds) Autoradiogram obtained after Reverse Northern blot hybridization of the spotted cloned gene fragments to a probe prepared by reverse transcription of 25 µg of total RNA purified from bud's abscission zone from both vanilla and lavender. Histogram summarizing densitometric analysis of Reverse Northern blots as described in (A) normalized to Rhβ-actin. Values represent means ± standard error of the mean (S.E.M.) or all genes investigated, the right cDNA size was amplified (see Table 2). Densitometric analysis was performed using ImageQuant TL (Amersham, GE Healthcare UK limited, Buckinghamshire, UK).

c) Ethylene signal transduction (RhCTR1-2) and transcription factors (RhEIN3, RhEIL)

The expression of ethylene signal transduction (RhCTR1-2) and transcription factors (RhEIN3, RhEIL) in bud's abscission zone of ethylene treated or non-treated 'Vanilla' and 'Lavender' plants was determined by reverse Northern dot blot. In 'Vanilla' ethylene treated plants, the expression of RhCTR1-2, RhEIN3, and RhEIL was much more abundant than in non-treated plants. On the contrary, in 'Lavender' ethylene non-treated plants, the expression of RhCTR1-2, RhEIN3, and RhEIL was higher than in treated plants (Fig. 21 A-B).

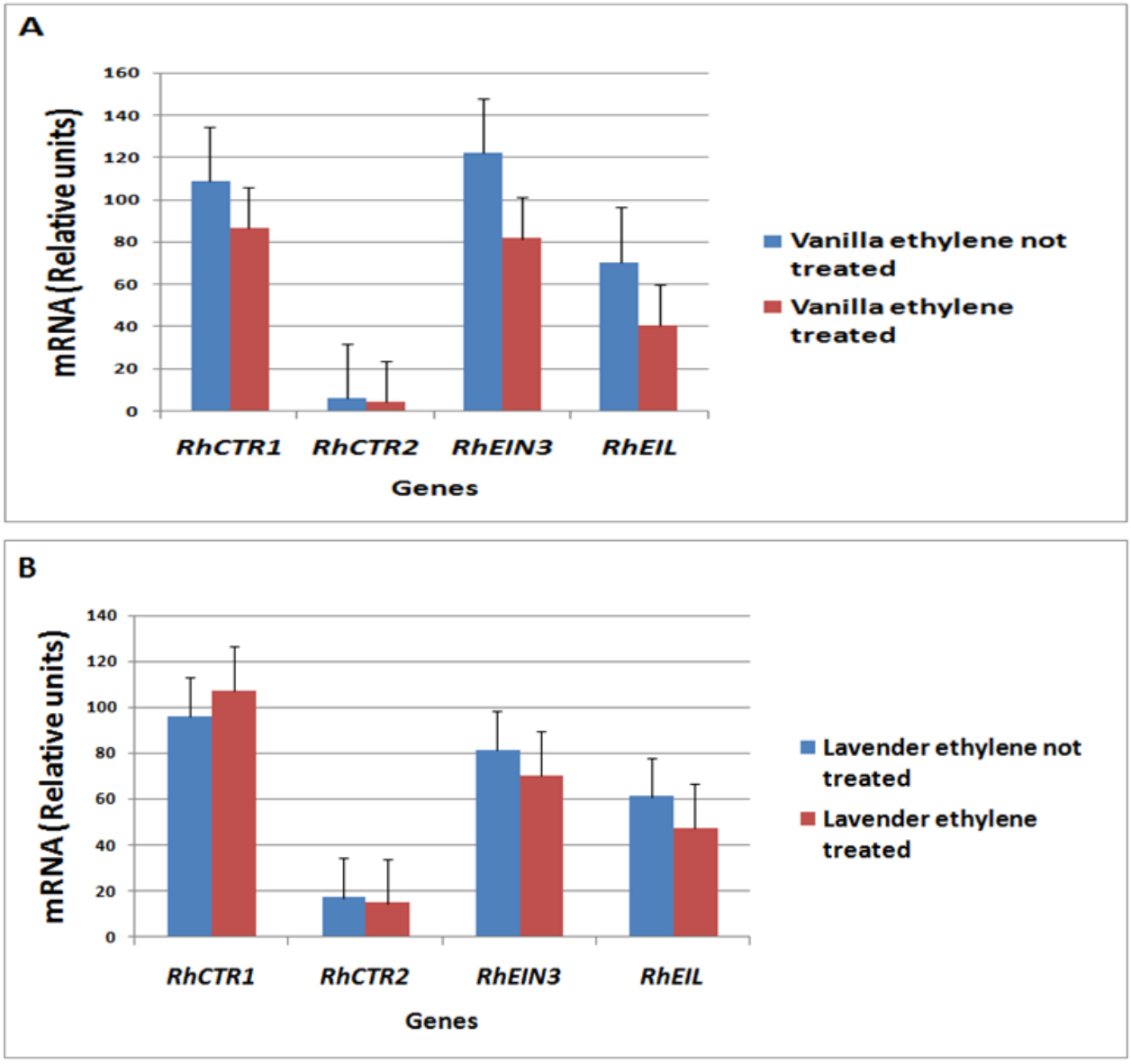


Figure 21.

Reverse Northern dot blots. Equal volumes of various amount of DNA (0.5, 0.1, or 0.02 µg) of the amplified genes fragments, together with various amount of Rh-β-actin DNA (0.5, 0.1, or 0.02 µg) were blotted on Hybond-N+ nylon transfer membrane using the micropipette. After denaturation, neutralization and DNA fixation, the filters containing the spotted DNA were hybridized to Dig-labelled cDNA probes from specified tissues. (A; petals, C; whole floral buds) Autoradiogram obtained after Reverse Northern blot hybridization of the spotted cloned gene fragments to a probe prepared by reverse transcription of 25 µg of total RNA purified from the full opening flower petals from both vanilla and lavender. (B; Petals, D; whole floral buds) Histogram summarizing densitometric analysis of Reverse Northern blots as described in (A) normalized to Rhβ-actin. Values represent means ± standard error of the mean (S.E.M.) or all genes investigated, the right cDNA size was amplified (see Table 2). Densitometric analysis was performed using ImageQuant TL (Amersham, GE Healthcare UK limited, Buckinghamshire, UK).

3.3.5 Expression Analysis by Northern blot hybridization

A PCR digoxigenin-labeled DNA probes for the internal control (*Rh-ß-actin*) was used to probe blots containing RNA from different rose tissues. The DIG-labeled Rh-ß-actin DNA hybridized to a single band at nearly 1.6 kb to RNA samples from different rose tissues (Fig. 22). A PCR Dig-labeled probes from different ethylene receptors, biosynthesis enzymes, transduction and transcription factors transcripts failed to hybridize to RNA samples (data not shown). Following Northern blot analysis with specific probes, the membrane was stripped and hybridized to a DIG-labeled *Rh-ß-actin* to check for even loading of the wells and complete transfer of the RNA (Fig. 22).

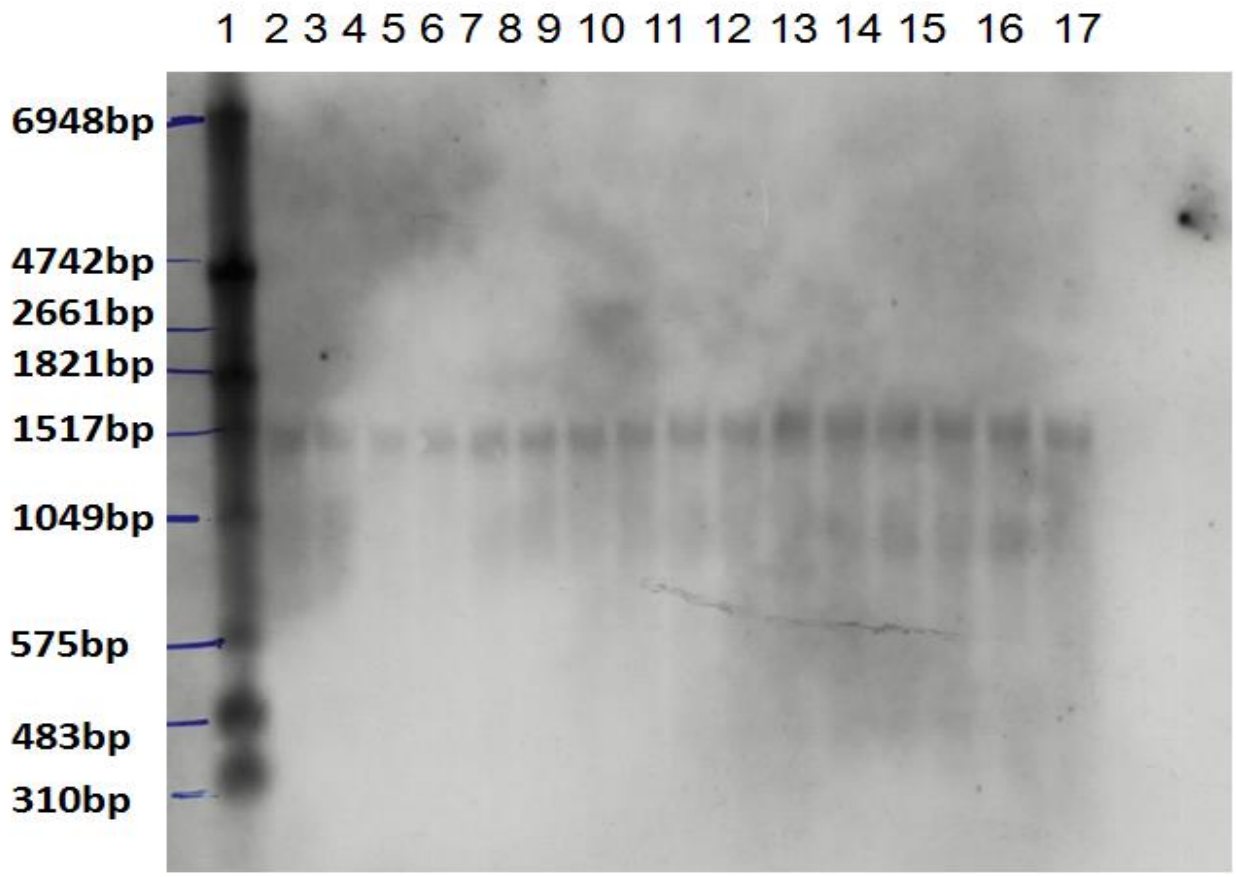
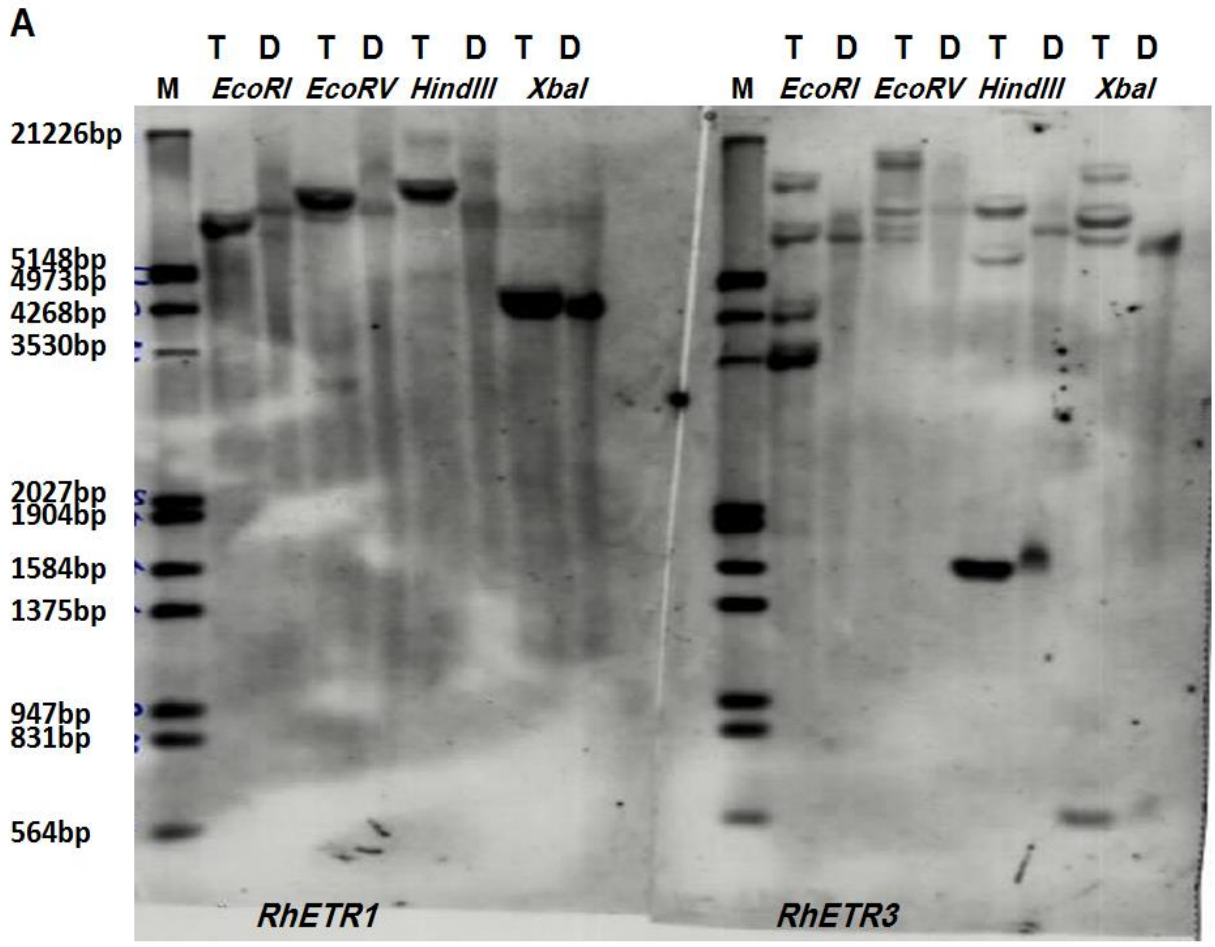
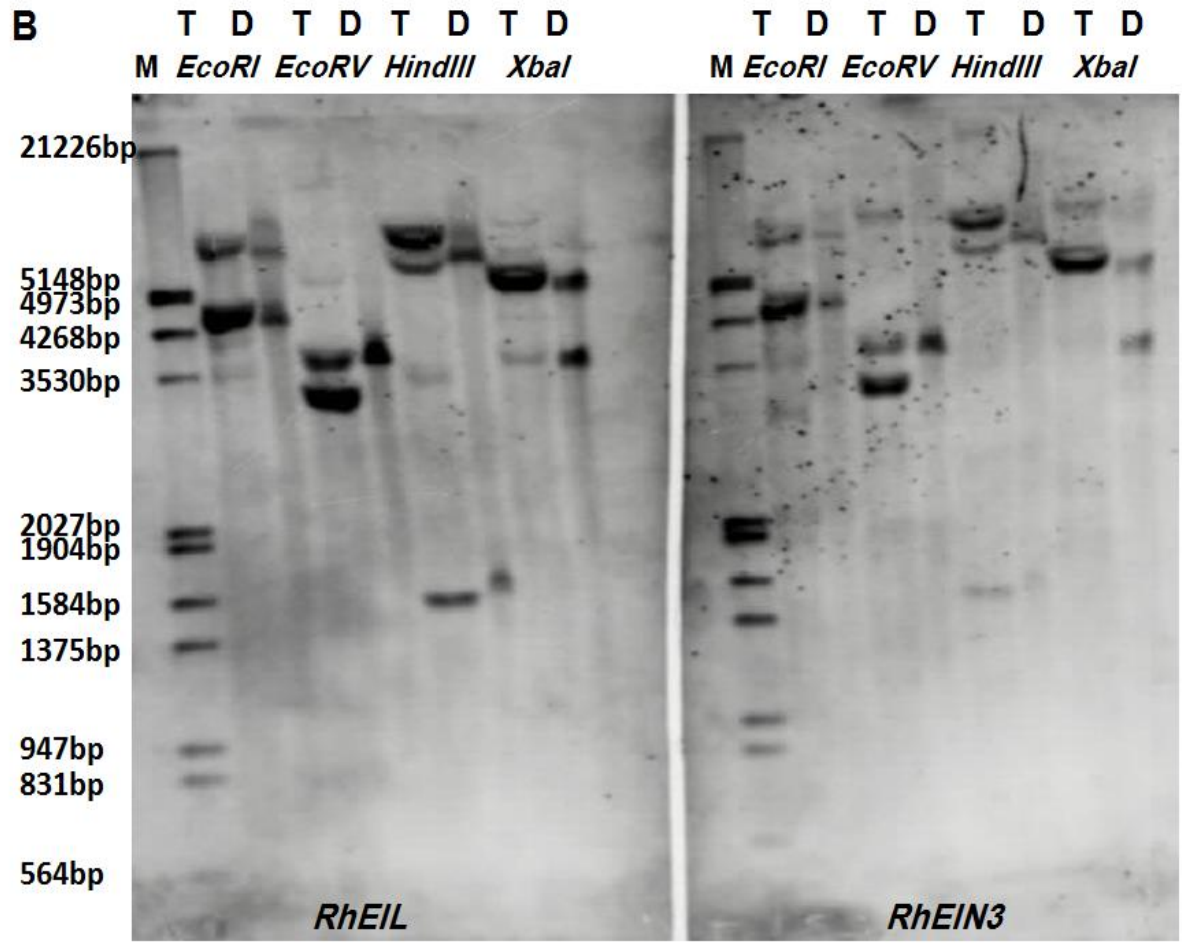


Figure 22.
Gene expression patterns Autoradiography of internal control Rh-ß-actin in different rose tissues and stages of developments by dig Northern blot analysis. RNA was isolated from the following tissues and separated on a formaldehyde/agarose gel as described previously in materials and methods: RNA molecular weight marker (lane 1), vanilla pistil stage 1(lane 2), vanilla pistil stage 2 (lane 3), vanilla pistil stage 3 (lane 4), lavender pistil stage 1 (lane 5), lavender pistil stage 2 (lane 6), lavender pistil stage 3 (lane 7), vanilla petal stage 1 (lane 8), vanilla petal stage 2 (lane 9), vanilla pistil stage 3(lane 10), lavender petal stage 1 (lane 11), lavender petal stage 2 (lane 12) lavender petal stage 3 (lane 13), Vanilla floral buds (lane 14), Lavender floral buds (lane 15), vanilla leaves (lane 16), lavender leaves (17).

3.4 Structure and copy number of selected genes

To examine the copy number of the genes that showed interested expression results, genomic DNA of 'Vanilla' and Diploid roses were digested with *EcoRI, EcoRV, HindIII,* and *XbaI*, and then hybridized with the specific coding sequence of each gene under high stringency condition. The result indicated that there were 2-4 specific hybridization bands of different sizes ranging from 15 to 0.5 kb (Fig. 23 A, B, and C). From the result obtained, it might be revealed that *RhETR3, RhETR1, RhEIN3, RhEIL, RhCTR1* and *RhCTR2* genes were all multi-copy genes in the rose genome. Additionally, these results showed differences between tetraploid and diploid species.




B
T D T D T D T D
M EcoRI EcoRV HindIII XbaI
T D T D T D T D
M EcoRI EcoRV HindIII XbaI
21226bp
5148bp
4973bp
4268bp
3530bp
2027bp
1904bp
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947bp
831bp
564bp
RhEIL
RhEIN3

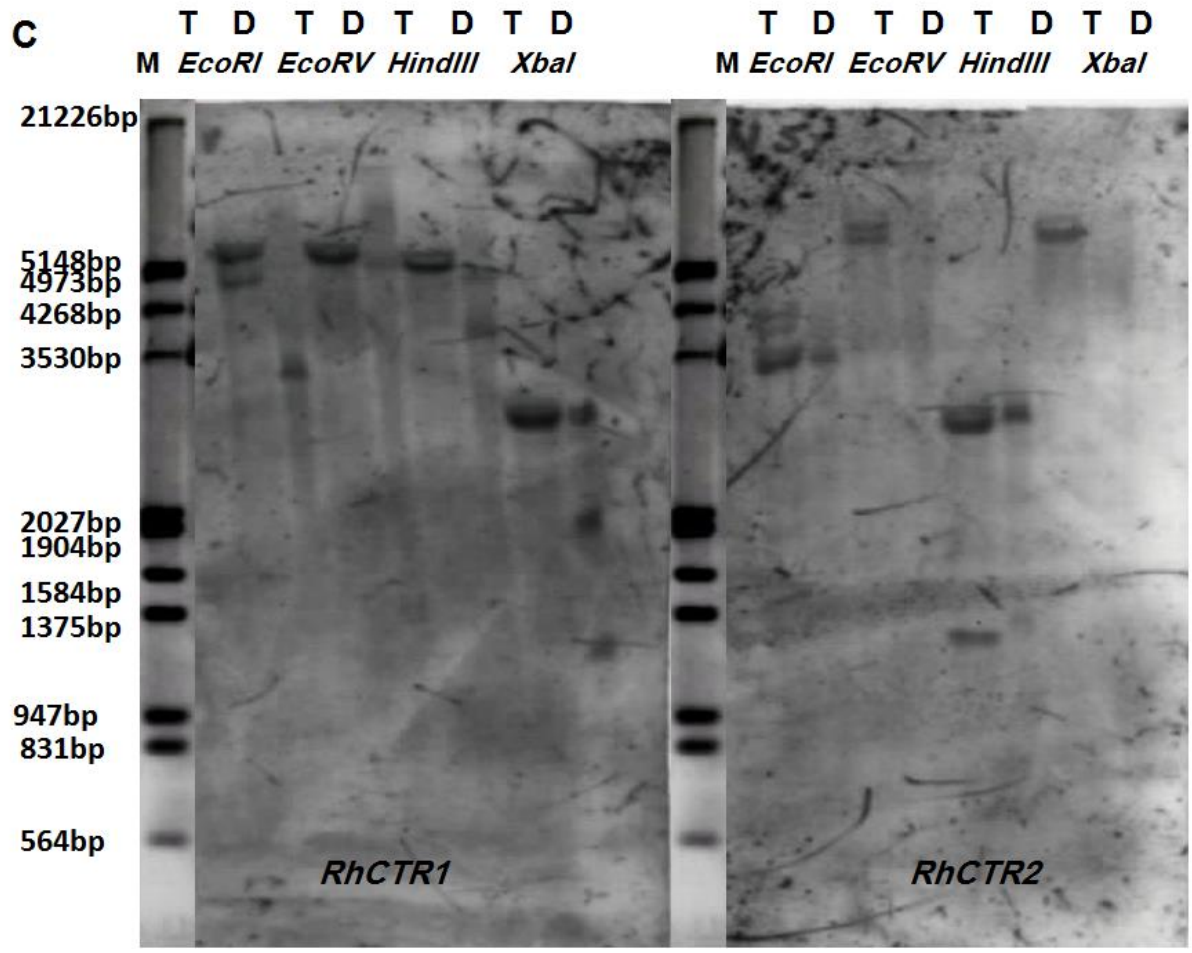

Figure 23.
Autoradiogram showing Non-radioactive southern blot analysis of two rose varieties, tetraploid (T) and Diploid (D). A genomic DNA (10µg per lane) was digested with EcoRI, EcoRV, HindIII, and XbaI and hybridized with the specific probes of indicated genes: DNA molecular weight marker (M), 'Vanilla' DNA (T) Diploid rose (D), RhETR1 and RhETR3 panel (A), RhEIL and RhEIN3 panel (B), RhCTR1 and RhCTR2 panel (C).

3.5 Identification of candidate SNPs markers associated with ethylene sensitivity by CAPS methods

3.5.1 In RhETR3

After cloning, and sequencing of a 661 bp fragment of RhETR3 from both 'Vanilla' and 'Lavender', both sequencers were compared to find single nucleotide polymorphisms. Five SNPs were detected from sequence alignments for the 661 bp cloned fragments (Fig. 24).

```
CLUSTAL W (1.83) multiple sequence alignment

RhETR3V         CACTGCTATAACGCTCATCACTCTCATTCCTTTGCTTCTCAAAGTCAAAGTGAGAGAATT 60
RhETR3L         CACTGCTATAACGCTCATCACTCTCATTCCTTTGCTTCTCAAAGTCAAAGTGAGAGAATT 60
                ************************************************************

RhETR3V         TATGTTGAAGAAGAAGACTTGGGACCTTGGAAGAGAGGTTGGGATTATAATGAGACAGAA 120
RhETR3L         TATGTTGAAGAAGAAGACTTGGGACCTTGGAAGAGAGGTTGGGATTATAATGAGACAGAA 120
                ************************************************************

RhETR3V         AGAGGCTGGAATGCATGTTCGAATGCTTACCCAAGAGATTCGCAAGTCTCTTGATAGACA 180
RhETR3L         AGAAGCTGGAATGCATGTTCGAATGCTTACCCAAGAGATTCGCAAGTCTCTTGATAGACA 180
                *** ********************************************************

RhETR3V         TACAATATTGTCCACAACCCTCTTTGAGCTATCTGAGACATTGGGTTTGCAGTACTGTGC 240
RhETR3L         TACAATATTGTCCACAACCCTCTTTGAGCTATCTGAGACATTGGGTTTGCAGTACTGTGC 240
                ************************************************************

RhETR3V         AGTTTGGATGCCTAATGAAATTAAAACGGAGATGATCCTGACCCATGAGTTGAAAGGGAG 300
RhETR3L         AGTTTGGATGCCTAATGAAATTAAAACGGAGATGATCCTGACCCATGAGTTGAAAGGGAG 300
                ************************************************************

RhETR3V         GAATTATTCTAATATGTACAACTTTTCTATACCAATAGGTGATCCAGATGTTGTACTTAT 360
RhETR3L         GAATTATTCTAATATGTACAACTTTTCTATACCAATAGGTGATCCAGATGTTGTACTTAT 360
                ************************************************************

RhETR3V         CAAAGGGAGTGATGGGGTCAACATCCTTGGGCCAGATTCAATACTCGTGTCCGGAAGCAG 420
RhETR3L         CAAAGGGAGTGATGGGGTCAACATCCTTGGGCCAGATTCAATACTCGTGTCTGGAAGCAG 420
                ************************************************ ********

RhETR3V         TGGTGATTTTGGTGAGCCGGGACCAGTAGCTGCAATACGGATGCCAATGCTTCGGGTTTC 480
RhETR3L         TGGTGATTTTGGTGAGCCGGGACCAGTAGCTGCAATACGGATGCCAATGCTTCGGGTTTC 480
                ************************************************************

RhETR3V         CAATTTCAAAGGGGGGACCCCTGAGTTCATCCAGACTTGTTATGCGATTTTGGTTCTGGT 540
RhETR3L         CAATTTCAAAGGGGGTACCCCTGAGTTGATCCAGACTTGTTATGCGATTTTGGTTCTGGT 540
                ************** *********** ********************************

RhETR3V         TCTCCCTGGTGGACAGCCTAGATCTTGGAGCAGCCAGGAACTTGAGATAATTAAGGTGGT 600
RhETR3L         TCTCCCTGGTGGACAGCCTAGATCTTGGAGCAGCCAGGAACTTGAGATAATTAAGGTGGT 600
                ************************************************************

RhETR3V         TGCCGATCAGGTGGCTGTGGCTTTATCCCATGCTGCAATCCTTGAAGAGTCCCAACTAAT 660
RhETR3L         TGCTGATCAGGTGGCTGTGGCTTTATCCCATGCTGCAATCCTTGAAGAGTCCCAACTAAT 660
                *** ********************************************************

RhETR3V         G 661
RhETR3L         G 661
```

Figure 24.
Multiple Sequence Alignment. A multiple sequence alignment was performed on partial nucleotide sequences of RhETR3 from the 'Vanilla' and 'Lavender'.. Stars"*" means that the nucleotides in that column are identical in all sequences in the alignment. Sequence gaps and nucleotide mismatches (candidate SNPs) are highlighted.

Among the five SNPs, three SNPs were detected in this partial DNA sequence of the RhETR3 alleles analyzed. The CAPS could distinguish RhETR3V and RhETR3L alleles could be a putative SNP marker to distinguish between rose cultivars regarding the ethylene sensitivity. The first CAPS was detected at position 496 bp with the use of Acc65I restriction enzyme (table 4).

Table 4. CAPS analysis of the RhETR3 gene in two cultivars of miniature roses 'lavender' and 'Vanilla'.

CAPS					Alleles			
Gene name	**Restriction Enzyme name**	**Times of Cuts**	**Recognition sequence**	**SNPs Positions**	**RhETR3-van**	**RhETR3-lav**	**RhETR3-van16**	**RhETR3-lav9**
RhETR3	*Acc65I*	1	5'-G^G T A C C-3' 3'-C C A T G^G-5'	496	No	Yes	No	No
	Kpn2I	1	5'-T^C C G G A-3' 3'-A G G C C^T-5'	412	yes	No	No	No

The expected result is to cut DNA at 494 bp position only in 'Lavender', because GGTACC is found only in Lavender sequence and repeated only one time. The second CAPS was detected at position 412 at which Kpn2I cut the DNA at position 410 in 'Vanilla' only. Different versions of alleles were screened by colony PCR followed by digestion the PCR product with acc65I and Kpn2I from 'Vanilla' and 'Lavender'. Firstly, colony PCR were conducted for 40 colonies, then the colonies that contain the insert were sub cultured on LB plates and colony PCR was done again with those positive colonies and then digested with acc65I and Kpn2I, (Fig. 25 & 26) then two different alleles were chosen, one from Vanilla and one from Lavender and sequenced, named as RhETR3-van16, RhETR3-lav9, (Fig. 27) shows the 4 different alleles alignment.

F1 plants were tested for the detected CAPS markers; Fig. 28 shows the amplification and digestion of CAPS marker in RhETR3 eight cultivars of potted roses. Amplification and *Acc65I* and *Kpn2I* digests were separated in 2.0% agarose gel. As seen from the (Fig. 28), the genotypes from F1 have reacted differently to the treatment of *Acc65I* and *Kpn2I*. The first 4 genotypes (128, 48, 131, and 67) considered as low sensitive to ethylene and the other 4 genotypes (50, 74, 143, 22) considered

as high sensitive to ethylene genotypes. From these results, it can be recognized that the RhETR3 CAPS markers generated by Acc65I or Kpn2I failed to assess the ethylene sensitivity cultivars.

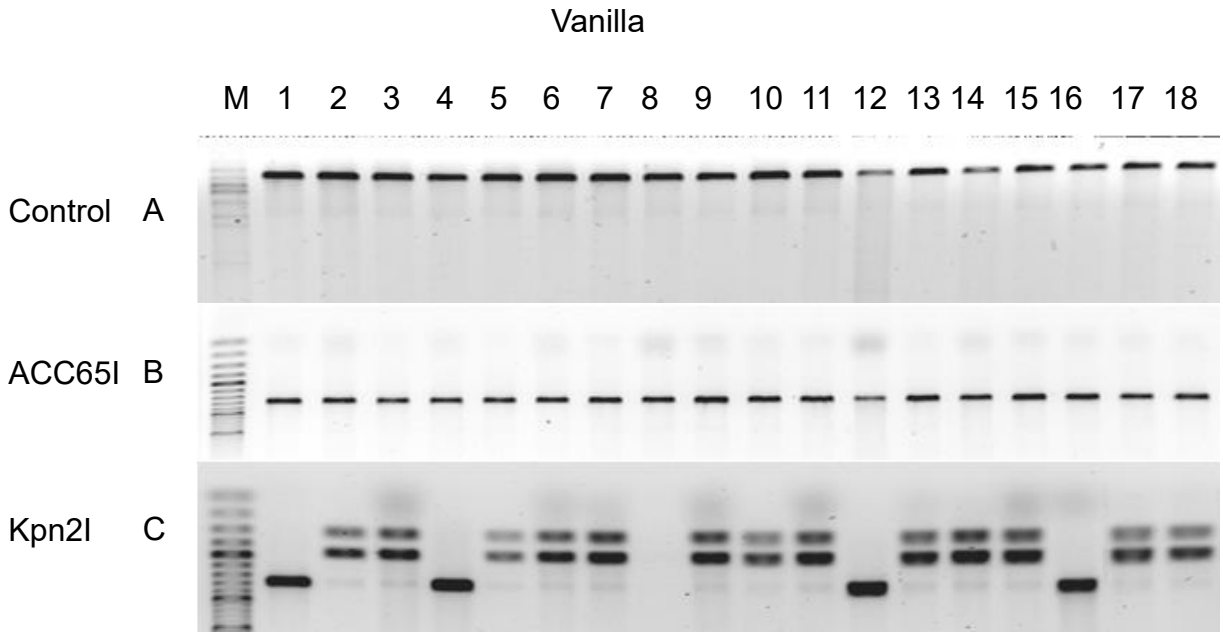

Figure 25.
Amplification and digestion of CAPS marker locus *RhET3* in 'Vanilla'. (A) Amplification of different RhETR3 colonies without treatment of restriction enzymes, (B) treated with ACC65I, (C) treated with *Kpn2I*. Lanes: M, 100bp ladder; 1-19 RhETR3 colonies.

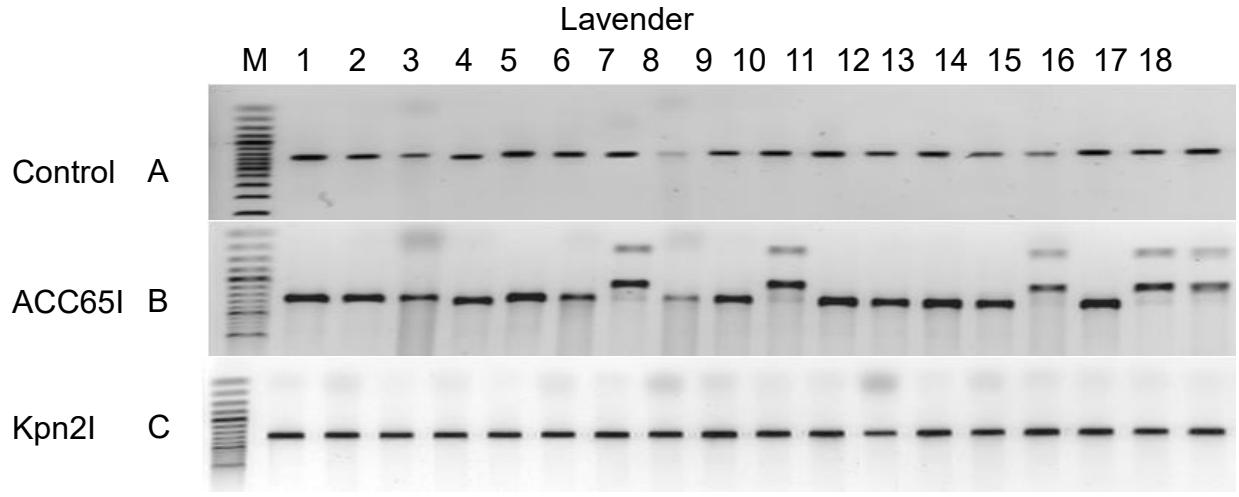

Figure 26.
Amplification and digestion of CAPS marker locus *RhET3* in 'Lavender'. (A) Amplification of different RhETR3 colonies without treatment of restriction enzymes, (B) treated with ACC65I, (C) treated with *Kpn2I*. Lanes: M, 100bp ladder; 1-19 RhETR3 colonies.

```
CLUSTAL W (1.83) multiple sequence alignment
RhETR3v#16      CCACTGCTATAACGCTCATCACTCTCATTCCTTTGCTTCTCAAAGTCAAAGTGAGAGAAT 60
RhETR3L         TCACTGCTATAACGCTCATCACTCTCATTCCTTTGCTTCTCAAAGTCAAAGTGAGAGAAT 60
RhETR3V         TCACTGCTATAACGCTCATCACTCTCATTCCTTTGCTTCTCAAAGTCAAAGTGAGAGAAT 60
RhETR3L#9       ACACTGCTATAACGCTCATCACTCTCATTCCTTTGCTTCTCAAAGTCAAAGTGAGAGAAT 60
                 ************************************************************

RhETR3v#16      TTATGTTGAAGAAGAAGACTTGGGACCTTGGAAGAGAGGTTGGGATTATAATGAGACAGA 120
RhETR3L         TTATGTTGAAGAAGAAGACTTGGGACCTTGGAAGAGAGGTTGGGATTATAATGAGACAGA 120
RhETR3V         TTATGTTGAAGAAGAAGACTTGGGACCTTGGAAGAGAGGTTGGGATTATAATGAGACAGA 120
RhETR3L#9       TTATGTTGAAGAAGAAGACTTGGGACCTTGGAAGAGAGGTTGGGATTATAATGAGACAGA 120
                ************************************************************

RhETR3v#16      AAGAAGCTGGAATGCATGTTCGAATGCTTACCCAAGAGATTCGCAAGTCTCTTGATAGAC 180
RhETR3L         AAGAAGCTGGAATGCATGTTCGAATGCTTACCCAAGAGATTCGCAAGTCTCTTGATAGAC 180
RhETR3V         AAGAGGCTGGAATGCATGTTCGAATGCTTACCCAAGAGATTCGCAAGTCTCTTGATAGAC 180
RhETR3L#9       AAGAGGCTGGAATGCATGTTCGAATGCTTACCCAAGAGATTCGCAAGTCTCTTGATAGAC 180
                **** ********************************************************

RhETR3v#16      ATACAATATTGTCCACAACCCTCTTTGAGCTATCTGAGACATTGGGTTTGCAGTACTGTG 240
RhETR3L         ATACAATATTGTCCACAACCCTCTTTGAGCTATCTGAGACATTGGGTTTGCAGTACTGTG 240
RhETR3V         ATACAATATTGTCCACAACCCTCTTTGAGCTATCTGAGACATTGGGTTTGCAGTACTGTG 240
RhETR3L#9       ATACAATATTGTCCACAACCCTCTTTGAGCTATCTGAGACATTGGGTTTGCAGTACTGTG 240
                ************************************************************

RhETR3v#16      CAGTTTGGATGCCTAATGAAATTAAAACGGAGATGATCCTGACCCATGAGTTGAAAGGGA 300
RhETR3L         CAGTTTGGATGCCTAATGAAATTAAAACGGAGATGATCCTGACCCATGAGTTGAAAGGGA 300
RhETR3V         CAGTTTGGATGCCTAATGAAATTAAAACGGAGATGATCCTGACCCATGAGTTGAAAGGGA 300
RhETR3L#9       CAGTTTGGATGCCTAATGAAATTAAAACGGAGATGATCCTGACCCATGAGTTGAAGGGGA 300
                ********************************************************** ****

RhETR3v#16      GGAATTATTCTAATATGTACAACTTTTCTATACCAATAGGTGATCCAGATGTTGTACTTA 360
RhETR3L         GGAATTATTCTAATATGTACAACTTTTCTATACCAATAGGTGATCCAGATGTTGTACTTA 360
RhETR3V         GGAATTATTCTAATATGTACAACTTTTCTATACCAATAGGTGATCCAGATGTTGTACTTA 360
RhETR3L#9       GGAATTATTCTAATATGTACAACTTTTCTATACCAATAGGTGATCCAGATGTTGTACTTA 360
                ************************************************************

RhETR3v#16      TCAAAGGGAGTGATGGGGTCAACATCCTTGGGCCAGATTCAATACTCGTGTCTGGAAGCA 420
RhETR3L         TCAAAGGGAGTGATGGGGTCAACATCCTTGGGCCAGATTCAATACTCGTGTCTGGAAGCA 420
RhETR3V         TCAAAGGGAGTGATGGGGTCAACATCCTTGGGCCAGATTCAATACTCGTGTCCGGAAGCA 420
RhETR3L#9       TCAAAGGGAGTGATGGGGTCACCATCCTTGGGCCAGATTCAGCACTCGTGTCTGGAAGCA 420
                ********************* ******************  ********* *******

RhETR3v#16      GTGGTGATTTTGGTGAGCCGGGACCAGTAGCTGCAATACGGATGCCAATGCTTCGGGTTT 480
RhETR3L         GTGGTGATTTTGGTGAGCCGGGACCAGTAGCTGCAATACGGATGCCAATGCTTCGGGTTT 480
RhETR3V         GTGGTGATTTTGGTGAGCCGGGACCAGTAGCTGCAATACGGATGCCAATGCTTCGGGTTT 480
RhETR3L#9       GTGGTGATTTTGGTGAGCCGGGACCAGTAGCTGCAATACGGATGCCAATGCTTCGGGTTT 480
                ************************************************************

RhETR3v#16      CCAATTTCAAAGGGGGGACCCCTGAGTTGATCCAGACTTGTTATGCGATTTTGGTTCTGG 540
RhETR3L         CCAATTTCAAAGGGGGTACCCCTGAGTTGATCCAGACTTGTTATGCGATTTTGGTTCTGG 540
RhETR3V         CCAATTTCAAAGGGGGGACCCCTGAGTTCATCCAGACTTGTTATGCGATTTTGGTTCTGG 540
RhETR3L#9       CCAATTTCAAAGGGGGGACCCCTGAGTTGATCCAGACTTGTTATGCGATATTGGTTCTGG 540
                *************** *********** ****************** **********

RhETR3v#16      TTCTCCCTGGTGGACAGCCTAGATCTTGGAGCAGCCAGGAACTTGAGATAATTAAGGTGG 600
RhETR3L         TTCTCCCTGGTGGACAGCCTAGATCTTGGAGCAGCCAGGAACTTGAGATAATTAAGGTGG 600
RhETR3V         TTCTCCCTGGTGGACAGCCTAGATCTTGGAGCAGCCAGGAACTTGAGATAATTAAGGTGG 600
RhETR3L#9       TTCTCCCTGGTGGACAGCCTAGATCTTGGAGCAGCCAGGAACTTGAGATAATTAAGGTGG 600
                ************************************************************

RhETR3v#16      TTGCTGATCAGGTGGCTGTGGCTTTATCCCATGCTGCAATCCTGAAGAGTCCCAACTA-T 659
RhETR3L         TTGCTGATCAGGTGGCTGTGGCTTTATCCCATGCTGCAATCCTGAAGAGTCCCAACTA-T 659
RhETR3V         TTGCCGATCAGGTGGCTGTGGCTTTATCCCATGCTGCAATCCTGAAGAGTCCCAACTAAT 660
RhETR3L#9       TTGCTGATCAGGTGGCTGTGGCTTTATCCCATGCTGCAATCCTGAAGAGTCCCAACTAAT 660
                **** ********************************************************* *
```

Figure 27.

Multiple Sequence Alignment. A multiple sequence alignment was performed on partial nucleotide sequences of four RhETR3 alleles from the 'Vanilla' and 'Lavender'... Stars"*" means that the nucleotides in that column are identical in all sequences in the alignment. Sequence gaps and nucleotide mismatches are highlighted.

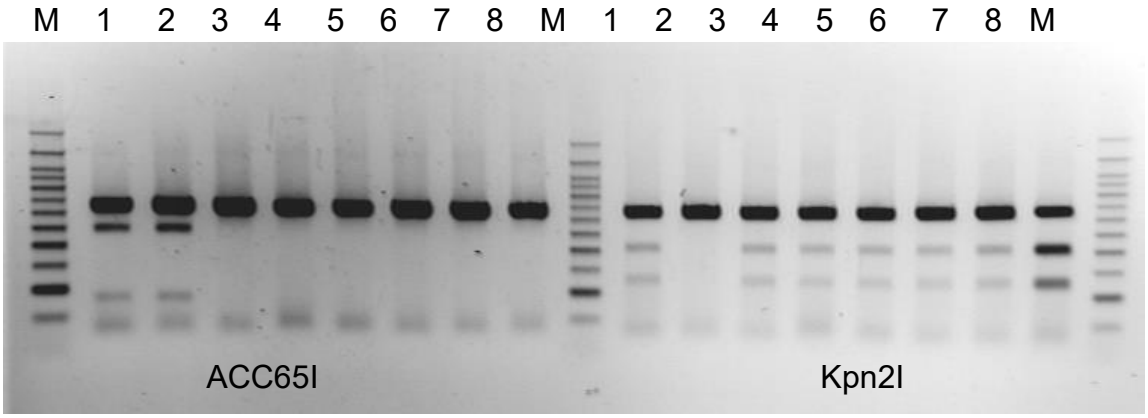

Figure 28.
Electrophoretic patterns produced by the CAPS method. Fragment sizes were calculated on the basis of sequence data. M: 100bp ladder. 1-8: F1 plants number. 128, 48, 131, 67, 50, 74, 143, 22 respectively.

3.5.2 In RhEIN3

New primer pair of RhEIN3 was designed in order to have more chance of SNPs occurring in the partial RhEIN3 sequence. The amplified PCR products from 'Vanilla' and 'Lavender' were cloned and then sequenced. 475 bp fragments of RhEIN3 from both 'Vanilla' and 'Lavender' were compared to find single nucleotide polymorphisms. Two SNPs were detected from sequence alignments for the 475 bp cloned fragments (Fig. 29).

```
CLUSTAL 2.0.5 multiple sequence alignment

RhEIN3-van-1      TCAAGCTCGTGGATTTGTGTATGGTATCATTCCTGAGAAGGGCAAGCCAGTAAGCGGTGC 60
RhEIN3-Lav-1      TCAAGCTCGTGGATTTGTGTATGGTATCATTCCTGAGAAGGGCAAGCCAGTAAGCGGTGC 60
                  ************************************************************

RhEIN3-van-1      TTCTGATAATATCAGAGCATGGTGGAAAGAAAAAGTGAAGTTTGATAAGAATGGCCCTGC 120
RhEIN3-Lav-1      TTCTGATAATATCAGAGCATGGTGGAAAGAAAAAGTGAAGTTTGATAAGAATGGCCCTGC 120
                  ************************************************************

RhEIN3-van-1      AGCCATAGACAAGTATGAAGCAGAGATTCTTGCCATGACTGATGCGGACAATAACCGAAA 180
RhEIN3-Lav-1      AGCCATAGACAAGTATGAAGCAGAGATTCTTGCCATGACTGATGCGGACAATAACCGAAA 180
                  ************************************************************

RhEIN3-van-1      TGGTAATTCCCAGACCATCCTCCAAGATCTACAAGATGCAACTCTTGGTTCTCTACTATC 240
RhEIN3-Lav-1      TGGTAATTCCCAGACCATCCTCCAAGATCTACAAGATGCAACTCTTGGTTCTCTACTATC 240
                  ************************************************************

RhEIN3-van-1      TTCATTGATGCAACATTGCGACCCCCCTCAAAGGAAGTATCCATTAGAAAAGGCAGTTCC 300
RhEIN3-Lav-1      TTCATTGATGCAACATTGCGACCCCCCTCAAAGGAAGTATCCATTAGAAAAGGCAGTTCC 300
                  ************************************************************

RhEIN3-van-1      GCCTCCTTGGTGGCCGACAGGAAATGAGGATTGGTGGATGAAATCAGGGTTACCCTGTGG 360
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RhEIN3-Lav-1      GCCTCCTTGGTGGCCGACAGGAAATGAGGATTGGTGGATGAAATCAGGGTTACCCTGTGG 360
                  ************************************************************

RhEIN3-van-1      TCAGAGTCCTCCTTATAAGAAGCCACATGACTTAAAGAAGATGTGGAAAGTTGGGGTGTT 420
RhEIN3-Lav-1      TCAGAGTCCTCCTTATAAGAAGCCACATGACTTAAAGAAGATGTGGAAAGTTGGGGTGTT 420
                  ************************************************************

RhEIN3-van-1      AACAGCTGTGATGAAGCACATGTCCCCTGATATTGCAAAGATAAGGCGGCATGTC 475
RhEIN3-Lav-1      AATAGCTGTGATAAAGCACATGTCCCCTGATATTGCAAAGATAAGGCGGCATGTC 475
                  ** ********* ****************************************
```

Figure 29.
Multiple Sequence Alignment. A multiple sequence alignment was performed on partial nucleotide sequences of RhEIN3 from the 'Vanilla' and 'Lavender'. Stars"*" means that the nucleotides in that column are identical in all sequences in the alignment. Sequence gaps and nucleotide mismatches (candidate SNPs) are highlighted.

Two SNPs were detected in this partial DNA sequence of the RhEIN3 alleles analyzed. The SNPs could distinguish RhEIN3-van and RhEIN3-lav alleles could be a putative SNP marker to distinguish between rose cultivars regarding the ethylene sensitivity. The first SNP was detected at position 423 bp with the use of *HpaI* restriction enzyme (table 5).

Table 5. CAPS analysis of the RhEIN3 gene in two cultivars of miniature roses 'lavender' and 'Vanilla'.

CAPS					**Alleles**			
Gene name	**Restriction Enzyme name**	**Times of Cuts**	**Recognition sequence**	**SNPs Positions**	**RhEIN3-van-1** **Frequency, Cut Positions**	**RhEIN3-Lav-1** **Frequency, Cut Positions**	**RhEIN3-van-2** **Frequency, Cut Positions**	**RhEIN3-Lav-2** **Frequency, Cut Positions**
RhEIN3	**HpaI**	1	5'-G T T^A A C-3' 3'-C A A^T T G-5'	423	1, 420	No Cut	1, 420	No Cut
	PvuII	1	5'-C A G^C T G-3' 3'-G T C^G A C-5'	425	1, 425	No Cut	1, 425	1, 425
	MboI	1 or 2	5'-^G A T C -3' 3'- C T A G^-5'	207	1,207	1,207	2,207 343	1,207

The expected result is to cut DNA at 420 bp position only in 'Vanilla', because GTT^AAC is found only in Lavender sequence and repeated only one time. The second CAPS was detected at position 425 at which *PvuII* cut the DNA at position 425 in 'Lavender' only. Different versions of alleles were screened by colony PCR followed by digestion the PCR product with *HpaI* and *PvuII* from 'Vanilla' and 'Lavender'. Firstly, colony PCR were conducted for 40 colonies, then the colonies

that contain the insert were sub cultured on LB plates and colony PCR was done again with those positive colonies and then digested with *HpaI* and *PvuII*, (Fig. 30 & 31) then two different alleles were chosen, one from Vanilla and one from Lavender and sequenced, named as RhEIN3-van2, RhEIN3-lav2. (Fig. 32) shows the 4 different alleles alignment. From these two alleles another CAPS was found in RhEIN3-van2 (table 5) in position 343 by using *MboI* which cuts other alleles one time, whereas it cuts RhEIN3-van2 two times and producing 3 fragments (207bp, 136 bp, and 132) (Fig. 30 & 31). F1 plants were tested for the detected CAPS markers; Fig. 33 shows the amplification and digestion of CAPS marker in RhEIN3 eight cultivars of potted roses. It shows agarose gel electrophoresis profiles of the PCR products with and without restriction enzyme treatment (*HpaI* and *PvuII*). when *HpaI* was used, it cut the PCR product produced from amplifying the plasmids (RhEIN3-van1 and RhEIN3-van2 as expected, it cut also both 'vanilla' and 'lavender' cultivars. such results were also obtained when F1 plants DNA are used as PCR templates leading to figure that these CAPS markers could not distinguish between cultivars. Same results also obtained when *PvuII* was used. The CAPS markers described here failed to be a rapid mean of identifying seedlings that sensitive or insensitive to ethylene.

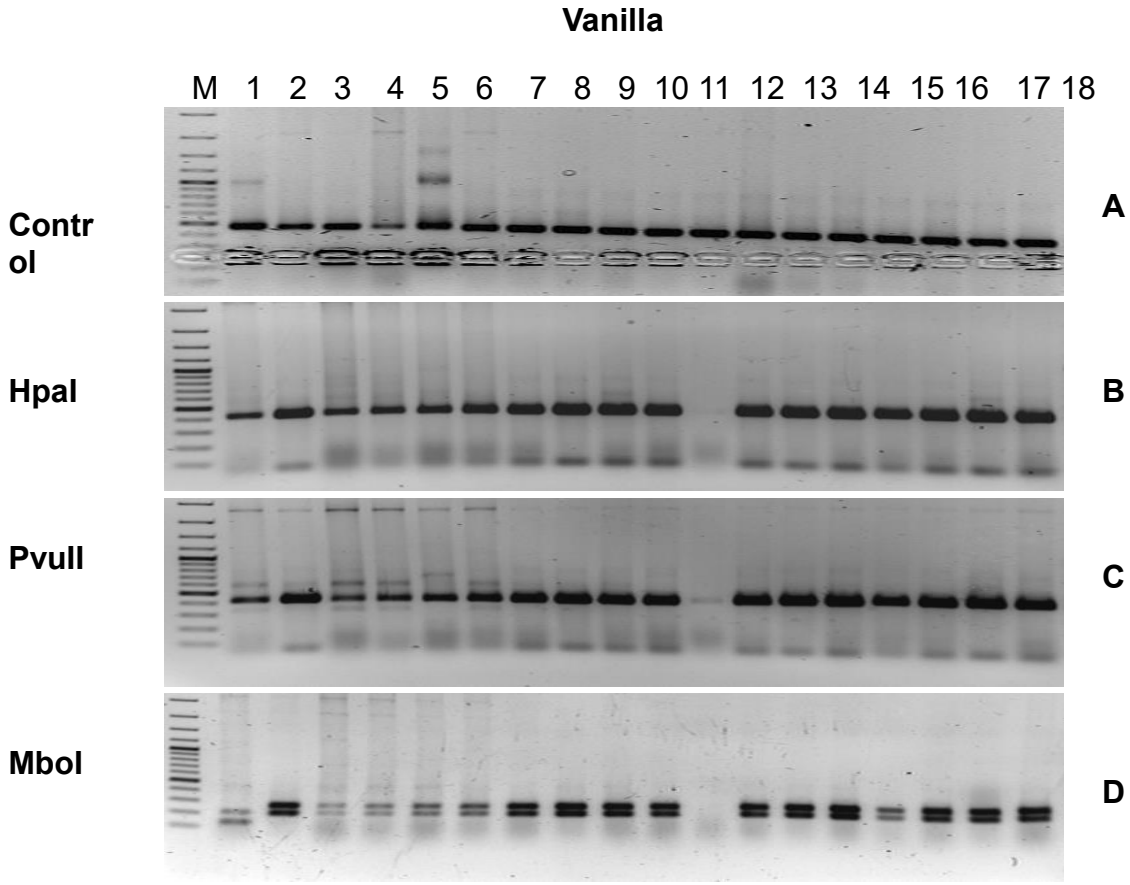

Figure 30.
Amplification and digestion of CAPS marker locus *RhEIN3* in 'Vanilla'. (A) Amplification of different RhETR3 colonies without treatment of restriction enzymes, (B) treated with HpaI, (C) treated with *PvuII*, (D) treated with *MboI*. Lanes: M, 100bp ladder; 1-18 RhEIN3 colonies

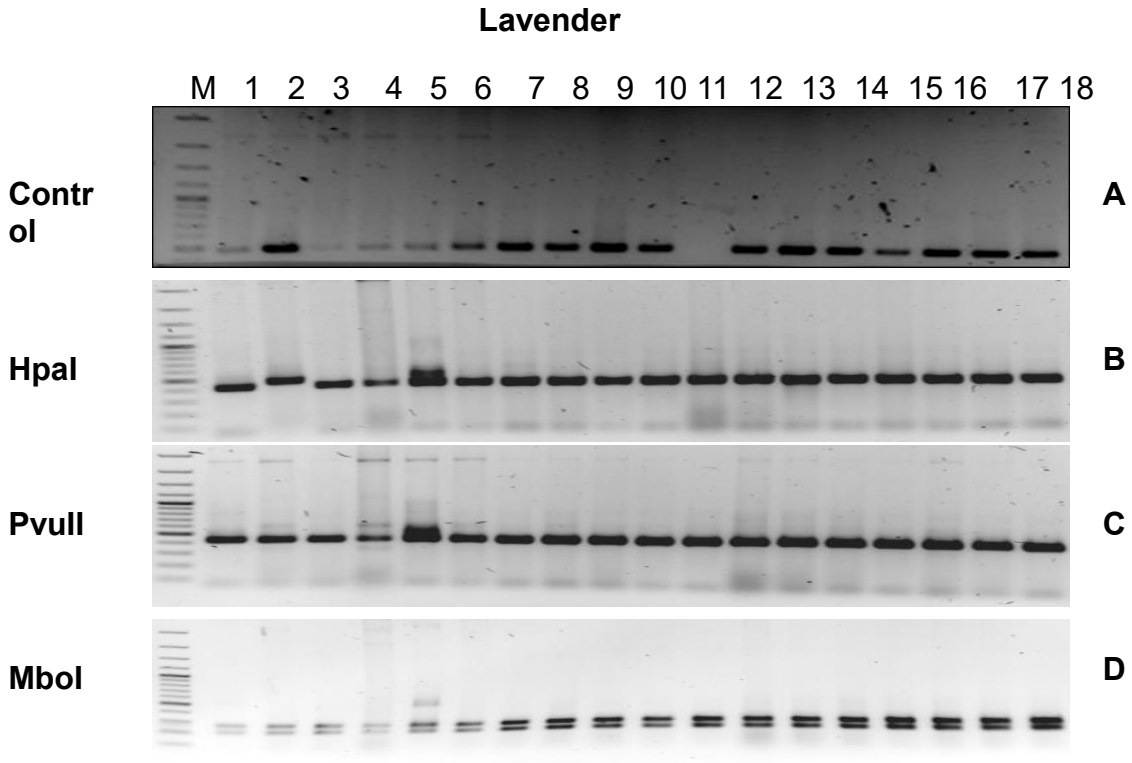

Figure 31.
Amplification and digestion of CAPS marker locus *RhEIN3* in 'Lavender'. (A) Amplification of different RhETR3 colonies without treatment of restriction enzymes, (B) treated with HpaI, (C) treated with *PvuII*, (D) treated with *MboI*. Lanes: M, 100bp ladder; 1-18 RhEIN3 colonies

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CLUSTAL 2.0.5 multiple sequence alignment


RhEIN3-Lav-1      TCAAGCTCGTGGATTTGTGTATGGTATCATTCCTGAGAAGGGCAAGCCAGTAAGCGGTGC 60
RhEIN3-Lav-2      TCAAGCTCGTGGATTTGTGTATGGTATCATTCCTGAGAAGGGCAAGCCAGTAAGCGGTGC 60
RhEIN3-van-1      TCAAGCTCGTGGATTTGTGTATGGTATCATTCCTGAGAAGGGCAAGCCAGTAAGCGGTGC 60
RhEIN3-van-2      TCAAGCTCGTGGATTTGTGTATGGTATCATTCCTGAGAAGGGCAAGCCAGTAAGCGGTGC 60
                  ************************************************************

RhEIN3-Lav-1      TTCTGATAATATCAGAGCATGGTGGAAAGAAAAAGTGAAGTTTGATAAGAATGGCCCTGC 120
RhEIN3-Lav-2      TTCTGATAATATCAGAGCATGGTGGAAAGAAAAAGTGAAGTTTGATAAGAATGGCCCTGC 120
RhEIN3-van-1      TTCTGATAATATCAGAGCATGGTGGAAAGAAAAAGTGAAGTTTGATAAGAATGGCCCTGC 120
RhEIN3-van-2      GTCTGATAACATCAGAGCATGGTGGAAAGAAAAAGTGAAGTTTGATAAGAATGGCCCTGC 120
                   ******** ************************************************

RhEIN3-Lav-1      AGCCATAGACAAGTATGAAGCAGAGATTCTTGCCATGACTGATGCGGACAATAACCGAAA 180
RhEIN3-Lav-2      AGCCATAGACAAGTATGAAGCAGAGATTCTTGCCATGACTGATGCGGACAATAACCGAAA 180
RhEIN3-van-1      AGCCATAGACAAGTATGAAGCAGAGATTCTTGCCATGACTGATGCGGACAATAACCGAAA 180
RhEIN3-van-2      AGCCATAGACAAGTATGAAGCAGAGATTCTTGCCATGACTGATGCGGACAATAACCGAAA 180
                  ************************************************************

RhEIN3-Lav-1      TGGTAATTCCCAGACCATCCTCCAAGATCTACAAGATGCAACTCTTGGTTCTCTACTATC 240
RhEIN3-Lav-2      TGGTAATTCCCAGACCATCCTCCAAGATCTACAAGATGCAACTCTTGGTTCTCTACTATC 240
RhEIN3-van-1      TGGTAATTCCCAGACCATCCTCCAAGATCTACAAGATGCAACTCTTGGTTCTCTACTATC 240
RhEIN3-van-2      TGGTAATTCCCAGACCATCCTCCAAGATCTACAAGATGCAACTCTTGGTTCTCTACTATC 240
                  ************************************************************

RhEIN3-Lav-1      TTCATTGATGCAACATTGCGACCCCCCTCAAAGGAAGTATCCATTAGAAAAGGCAGTTCC 300
RhEIN3-Lav-2      TTCATTGATGCAACATTGCGACCCCCCTCAAAGGAAGTATCCATTAGAAAAGGCAGTTCC 300
RhEIN3-van-1      TTCATTGATGCAACATTGCGACCCCCCTCAAAGGAAGTATCCATTAGAAAAGGCAGTTCC 300
RhEIN3-van-2      TTCATTGATGCAACATTGCGACCCCCCTCAAAGGAAGTATCCATTAGAAAAGGCAGTTCC 300
                  ************************************************************

RhEIN3-Lav-1      GCCTCCTTGGTGGCCGACAGGAAATGAGGATTGGTGGATGAAATCAGGGTTACCCTGTGG 360
RhEIN3-Lav-2      GCCTCCTTGGTGGCCGACAGGAAATGAGGATTGGTGGATGAAATCAGGGTTACCCTGTGG 360
RhEIN3-van-1      GCCTCCTTGGTGGCCGACAGGAAATGAGGATTGGTGGATGAAATCAGGGTTACCCTGTGG 360
RhEIN3-van-2      GCCTCCTTGGTGGCCGACAGGAAATGAGGATTGGTGGATGAGATCAGGGTTACCCTGTGG 360
                  ************************************* ******************

RhEIN3-Lav-1      TCAGAGTCCTCCTTATAAGAAGCCACATGACTTAAAGAAGATGTGGAAAGTTGGGGTGTT 420
RhEIN3-Lav-2      TCAGAGTCCTCCTTATAAGAAGCCACATGACTTAAAGAAGATGTGGAAAGTTGGGGTTTT 420
RhEIN3-van-1      TCAGAGTCCTCCTTATAAGAAGCCACATGACTTAAAGAAGATGTGGAAAGTTGGGGTGTT 420
RhEIN3-van-2      TCAGAGTCCTCCTTATAAGAAGCCACATGACTTAAAGAAGATGTGGAAAGTTGGGGCGTT 420
                  ********************************************************  **

RhEIN3-Lav-1      AATAGCTGTGATAAAGCACATGTCCCCTGATATTGCAAAGATAAGGCGGCATGTC 475
RhEIN3-Lav-2      AACAGCTGTGATAAAGCACATGTCCCCTGATATTGCAAAGATAAGGCGGCATGTC 475
RhEIN3-van-1      AACAGCTGTGATGAAGCACATGTCCCCTGATATTGCAAAGATAAGGCGGCATGTC 475
RhEIN3-van-2      AACAGCTGTGATAAAGCACATGTCCCCTGATATTGCAAAGATAAGGCGGCATGTC 475
                  ** ********* ****************************************
```

Figure 32.

Multiple Sequence Alignment. A multiple sequence alignment was performed on partial nucleotide sequences of four RhEIN3 alleles from the 'Vanilla' and 'Lavender'. Stars"*" means that the nucleotides in that column are identical in all sequences in the alignment. Sequence gaps and nucleotide mismatches are highlighted.

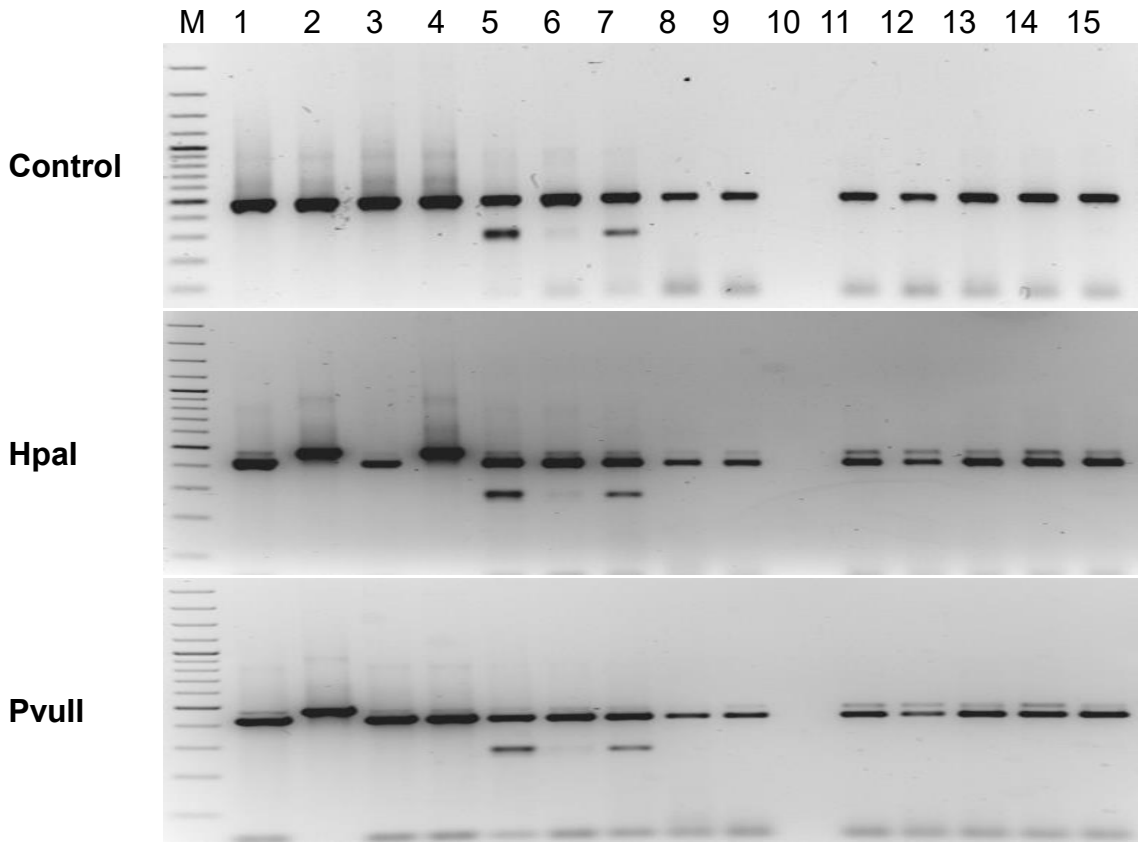

Figure 33.
Electrophoretic patterns produced by the CAPS method. Fragment sizes were calculated on the basis of sequence data. M: 100bp ladder. 1: plasmid DNA RhEIN3-van-1, 2: plasmid DNA RhEIN3-lav-1, 3: plasmid DNA RhEIN3-van-2, 4: RhEIN3-lav-2, 5: 'Vanilla', 6: 'Lavender', 7: diploid rose, 8-15: F1 plants number. 128, 48, 131, 67, 50, 74, 143, 22 respectively.

CHAPTER 4

DISCUSSION

4. DISCUSSION

4.1 Crossing Experiments:

Selfing ‘Vanilla’ cultivar and backcrosses with F1 plants obtained from a cross between ‘Vanilla’ × ‘Lavender’ would allow genetic manipulations of this long-lasting flowers cultivar and would assist forward physiological and molecular research in those plants with homogeneous genetic backgrounds. During the period of this research, there was a general difficulty in self-crossing and backcrossing ‘Vanilla’, but this problem was solved after investigating the reasons that prevents the success of the crosses. Unfortunately, the seed that resulted from these self- and back-crosses still in process of germination and was not used in this research due to the limitation of time since the success of getting these seeds was at the end of the research period, but they will be used in next research project.

Reasons that led to failure of self-pollination were investigated in ‘Vanilla’. Although previous crosses between ‘Vanilla’ × ‘Lavender’ were successful, self-pollination crosses had failed. Possible explanations for this failure can be due to several reasonability causes include, presence of self-incompatibility, poor-quality pollen, environmental conditions, and flower malformation.

The presence of self-incompatibility has been verified in *R. rugosa* (Ueda et al., 1996), in some diploid rose genotypes (Nybom et al., 2004; Debener et al., 2003; Debener, 1999).

Erlanson (1929) discussed the sterility in wild roses and in some species hybrids; he described the sterility in diploid and tetraploid species and the variation in pollen sterility in the same individual in one season and from season to season. Ishimizu et al. (1998) reported that the S-RNase which associated with gametophytic self-incompatibility in the Rosacea is responsible for S-allele-specific recognition in the self-incompatible reaction. De Nettancourt (1977) reported that tetraploid species, or artificially induced tetraploids, having monofactorial gametophytic self-incompatibility, usually display a self-compatible habit. Other members in Rosaceae also showed

self-incompatibility in some almond cultivars (Socias et al., 1976; Tao et al. 1997), apple (Sassa et al. 1994), Japanese pear (Ishimizu et al. 1998) and others more. Ueda and Akimoto (2001) deduced that a self-incompatibility system greatly exists in the genus *Rose,* distinctively in diploid species. Poor-quality pollen could also be another reason for the failure of 'vanilla' selfing. Initial in vitro germination of pollen grains from 'Vanilla' showed very poor quality of pollen, very low germination percentage and the germination pollen tube was very short. Cole (1917) reported that the species of *Rosa* are characterized by a large amount of abortive pollen and also by great variability. In the last year spring, very nice full open 'Vanilla' flowers with high number of pollen grains were noticed in the greenhouse, these plants were young and propagated to be used in another experiment. Pollen from these plants has been tested for germination in vitro and produced high germination percentage. Many researchers reported that nutrition conditions and environmental factors affect the ability of pollen to germinate and thus capable of fertilization. Gudin (1992) studied the effect of bud chilling on reproductive fertility in roses; he reported that the cold pre-treatment effect improves the in vitro germination of the pollen tubes and determine a specific morphology of the styles and thus influence the fertility. In another research, Gudin et al. (1991) indicated that the length of the pollen tubes emitted *in vitro* represents a valuable criterion of pollen fertilizing quality in roses. From our results, we can figure that the failure of self- and back-crosses made between 'Vanilla' and F1 genotypes was due to variation in pollen quality and viability rather than incompatibility.

4.2 Gene Expression Studies:

The beginning stage of this project showed the challenge of performing molecular biological experiments with rose tissues. Applying molecular biological methods to the miniature roses required a lot of methods testing, modifying, optimizing and developing of new concepts and

protocols, due to the fact that the existing methods were not directly feasible for the different tissues of roses. In addition to, the limiting budget and time contribute also to this challenge.

4.2.1 RNA isolation

The success of RNA isolation can usually be the first key factor of gene expression studies, especially when an adequately large quantity of good quality RNA is needed. The causes of difficulties when isolating RNA from roses are primarily the presence of high levels of secondary metabolites such as polyphenols, polysaccharides and other compounds (Salzman et al.1999; Malnoy et al., 2001; Gasic et al., 2004). These compounds can bind (phenolic compounds) and/or co-precipitate (polysaccharides) with nucleic acids and thus reduce RNA yield and produce poor quality RNA or completely no RNA, they can also irreversibly react with nucleic acids and cause problems in subsequent applications such as cDNA synthesis, and/or Northern hybridization (Gehrig et al. 2000; Salzman et al.1999; Tesniere and Vayda, 1991).

For extracting RNA from rose, several methods and kits were tested at the beginning of the project. Some of them gave low yields of varying contaminated and /or impure RNA, while others were simply unsuccessful. A hot-borate method of Wilkins and Smart (1996) was further modified (see section 2.5) for optimizing the yield and purity of RNA from rose tissues confirmed to be most successful and used to isolate high yield of good quality RNA, this method, was developed to isolate high-quality RNA from especially recalcitrant plant species having within tissues plentiful levels of phenolics, polysaccharides or other secondary metabolites (Wilkins and Smart, 1996).

4.2.2 Gene expression techniques

Gene expression or the process by which information encoded in a gene is transcribed into RNA, and then typically into protein can help to find the possible biological functions of investigated target genes. Expressed genes composed of genes that are transcribed into mRNA and then

translated into protein, as well as genes that are transcribed into types of RNA such as tRNA and rRNA that are not translated into protein. Gene expression is a highly specific process in which a gene is turned on at a definite time of developmental stage, and thus begins production of its protein. To study the abundance of transcripts of the ethylene receptor genes, ethylene biosynthesis enzymes genes, signal transduction and transcription genes, we first used reverse transcriptase PCR, and then tried to confirm it through non-radioactive Northern blot hybridization. Northern analysis is the only approach available that can determine the molecular weight of an mRNA species. It is also the least sensitive. Unfortunately, non-radioactive Northern blot hybridization was unable to produce any specific hybridization signals for the investigated genes, only in case of internal control gene *Rh-β-actin*, we obtained a weak uniform signal. However, the same probes were used in southern blot hybridization which produced clear useful results, indicating that there were no problems with probes. We tried many Northern blot protocols and modifications but without any success. A method described by Jaakola et al. (2001) used cDNA instead of RNA in Northern blotting, depending on the fact that cDNA is more stable than RNA and cancelation of any RNase contamination may arise. This method of cDNA blotting was used in this project but without any success to get any hybridization signals.

Several critical steps control the success of non-radioactive Northern blotting, these steps include: quality and quantity of RNA used, blotting method of RNA from gel to membrane, RNA immobilization to the membrane, quality of the labelled probe and method of detection, washing steps and stringency. Therefore, each of these steps must be empirically optimized which implies a relatively difficult and costly standardization period (Solanas et al. 2001). In the present study, we have tested and optimized all steps and conditions of non-radioactive Northern procedure but without any success and no hybridization signals were detected, suggesting that the investigated genes are expressed at levels below the limit of detection, or simply the failure of method due to

unknown reasons. If we consider the method as unsuccessful, then the failure of using DIG-labeled and detection system to detect a specific hybridization signals for the investigated RNA is likely due to the minor residues of secondary metabolites bound to the RNA samples, since these possible residues still did not interfere with the enzyme activity during reverse-transcription (Jaakola et al. 2001). Nevertheless, similar non-radioactive Northern hybridization blotting has been found to give variable results with roses (Müller et al., 2000a; Ma et al. 2005). Other explanations that might prevent the non-radioactive Northern blot to succeed include: specific activity of the probe is too low, Stringency is too high, RNA is degraded by RNAses during the procedure.

In the present study, expression of, ethylene receptor genes, ethylene biosynthetic enzymes genes and ethylene signal transduction & transcription genes were compared in two cultivars of miniature roses with different postharvest life, 'Vanilla' a long-lasting flowers cultivar and 'Lavender' a short-lasting flowers cultivar. The expression of in F1 progenies of 'Vanilla' and 'Lavender' was also evaluated in three tissues: petals, floral buds, and leaves in four selected genotypes classified in two groups relying on their responses to exogenous ethylene application, the first 2 genotypes (48 and 131) considered as low sensitive to ethylene and the other 3 genotypes (50 and 74, and) considered as high sensitive to ethylene genotypes (Ahmad et al. 2009).

To validate the expression results obtained by RT-PCR, A variant of the Northern blot hybridization known as the reverse Northern dot-blot hybridization technique were carried out, in which the membrane was hybridized with DIG-labelled cDNA probes that were reverse transcribed from the total RNA. This method was used as an alternative to conventional Northern blot. Such a method offers the advantage of being more sensitive than conventional Northern blotting (Liao and Freedman, 1998; Carginale et al., 2002). Reverse Northern dot-blot hybridization produced clear and evident results. These results further support the expression results obtained by RT-PCR analysis.

4.2.2.1 Expression of ETRs

Several molecular genetic researches have been conducted to get a deeply knowledge about ethylene response, resistance, perception and signal transduction pathways by utilizing the information available from extensive work carried out in Arabidopsis which have approved that ethylene perception in plants is mediated by a family of receptors. In order to understand how plants systematize their ethylene sensitivity, ethylene receptor genes should be identified, characterized, and cloned. Ethylene receptor genes has been isolated from several plant species, in roses, four ethylene receptor genes namely *RhETR1, RhETR2, RhETR3,* and *RhETR4* have been previously isolated in miniature roses (Müller et al., 2000a; Müller et al., 2000b), plus another one ethylene receptor, which has been lately isolated and identified as *Rh-ETR5* (Tan et al. 2006).

Expression analysis of ethylene receptors genes by RT-PCR showed that the expression level of *RhETR1* mRNA in carpels, petals, and floral buds of 'Vanilla' was higher than in 'Lavender'. Reverse Northern dot blot further supported the above findings in two tissues, petals of a full opening flower stage and in closing floral buds. In 'Vanilla' higher *RhETR1* mRNA abundance was detected in the second stage of carpels and 3rd stage of petals, whereas it was highly accumulated in mostly in carpel first stage and in leaves of 'Lavender'. Our results are not in agreement with those reported earlier by Müller et al., (2000a) as they reported that the expression of RhETR1 in the cultivar 'Bronze' with a short flower life was clearly higher than in the long-lasting 'Vanilla' in petals. In addition to, they reported that the highest expression of RhETR1 transcripts was at bud stage 'Bronze' or in young open flowers in 'Vanilla', While our results showed variable differences between the two cultivars deepening on the tissue, the expression of RhETR1 in bud stage was lowest in 'Lavender' while it was high in almost all stages investigated in 'Vanilla' with highest mRNA levels in full open flowers stages in pistil. In addition, we found that the expression of RhETR1 in leaves of 'Lavender' was clearly higher than in the leaves of

'Vanilla', these results are partly supported by the findings of Buanong et al. (2005) when she reported that, in free air environment (control treatments), RhETR1 was expressed in leaves of both 'Vanilla' and 'Lavender', however it was not expressed in petals. She did not compare the expression between 'Vanilla' and 'Lavender', but she reported that after exogenous ethylene treatment, RhETR1 expression in leaves was highly accrued in 'Lavender' than in 'Vanilla', treatment of exogenous ethylene promoted RhETR1 expression in petal and resulted in strong expression in leaves of both cultivars which is consistent with the previous finding of Müller at al. (2000a) in petals. In cut roses petals, and without ethylene treatment Tan et al. (2006) have detected the expression of Rh-ETR1 in cultivar 'Samantha' whereas; it was undetectable in cultivar 'Kardinal' even after ethylene treatment, which escalated the expression in cv. 'Samantha', the up-regulation of ETR, after ethylene treatment indicates that the sensitivity to ethylene is down regulated in 'Samantha' but not in 'Kardinal'. Müller et al. (2000a) concluded also that treatment of low ethylene concentrations resulted in an up-regulation of RhETR1 in flowers of both cultivars ('Bronze' and 'Vanilla'). On the other hand, both 'Samantha' and 'Kardinal' were sensitive to exogenous ethylene. However, remarkable different tendency of flower morphological changes was observed during and after ethylene treatment which accelerated flower opening, senescence, and caused petal abscission in 'Samantha', while it inhibited flower opening severely, resulted in bull flower, and even wilting directly in 'Kardinal' Lui et al. (2005). Several research studies have focused on ethylene receptor genes and the relationships with postharvest life in some other ornamental plants. For example, in carnation (*Dianthus caryophyllus* L.), the DC-ERS2 and DC-ETR1 mRNAs were expressed in considerable amounts in petals, ovaries, and styles of the flower at the full-opening stage (Shibuya et al., 2002), they reported also that DC-ETR1 mRNA showed no or little changes in any of the tissues during senescence. They have been also observed that the expression of DC-ERS2 and DC-ETR1 in petals decreased inversely to the increase of ethylene

production in flowers indicating that DC-ERS2 and DC-ETR1 are not positively regulated by ethylene.

The expression of RhETR1 in F1 progenies of 'Vanilla' and 'Lavender' was also evaluated in three tissues: petals, floral buds, and leaves. Generally, RhETR1 was expressed in all tissues, the results suggested highest accumulation of RNA in petals tissues over floral buds and leaves of all genotypes except in one. In floral buds and leaves, Different expression patterns were obtained. Expression patterns of plant genotype Nr. 48 from the ethylene low sensitive groups behaved the same as 'Vanilla', thus supporting the previous our results in 'vanilla'. On the other hand, plant genotype Nr. 74 from the ethylene high sensitive groups behaved as 'Lavender'. Further gene expression comparisons between genotypes 48 and 74 were performed by reverse Northern dot plot, which revealed that RhETR1 was slightly higher in genotype 74 than in genotype 48; therefore, we cannot confirm that F1 plants reacted as parents regarding the expression of RhETR1. RhETR2 was generally very low expressed in 'Vanilla' and 'Lavender' as RT-PCR results showed, particularly in tissues of carpels, petals and floral buds. 'Vanilla' exhibited a little more RhETR2 transcripts abundance than in same tissues in 'Lavender' which showed very low or undetectable expression except in leaves, in which more RhETR2 mRNA was detected than in 'Vanilla'. Reverse Northern dot blot confirmed the above results peculiarly in petals and leaves. Our results are not in agreement with what Müller et al. (2000b) published, when they stated that the expression of RhETR2 in petals of the two cultivars ('Bronze' and 'Vanilla') is constitutive during flower senescence, even though the transcript abundance differed between 'Bronze' and 'Vanilla' cultivars with different postharvest performance, which was Higher in Bronze than in 'Vanilla' in the three stage is development. Buanong et al. (2005) indicated that RhETR2 was expressed only in leaves but not in petals of both 'vanilla' and 'Lavender' in control plants which receive no ethylene treatment. In geranium two ethylene receptor genes, PhETR1 and PhETR2, have been

isolated (Dervinis et al., 2000), PhETR1 and PhETR2 were expressed at moderate levels in leaves, pedicels, sepals, pistils and petals, and at very low levels in roots. They indicated also that the levels of PhETR1 and PhETR2 transcripts were not up-regulated by exogenous ethylene treatment during flower senescence. However, Mutui et al. (2007) found that PhETR2 was strongly and constitutively expressed in all plant tissues (roots, stems, leaves, floral buds, petals, and pistils) irrespective of developmental stage or treatment applied. These findings are consistent with Wang et al. (2007) who have cloned and characterized two cDNAs encoding ethylene receptors namely PhERS1 and PhETR2 from petunia. They found that these two genes are constitutively expressed in stem, root, flower floral buds, and flowers. They reported also that the expression of PhETR2 in leaves is either less than the level of detection by Northern blot or there is no expression in leaves at all, but when they use RT-PCR to analyze the expression pattern of both PhERS1 and PhETR2, they found that these two genes are expressed at low levels in leaves compared with that in the other organs. Similar results were obtained in persimmon (*Diospyros kaki* Thumb.), the authors indicated that DkETR1 and DkETR2 transcripts was so low that it could not be detected by Northern blot analysis fruit during fruit development and ripening, they have performed cDNA Southern blot analysis was in order to detect the expression at a more sensitive level. DkETR1 exhibited a constitutive basal level expression throughout different stages; whereas DkETR2 showed a similar expression pattern to that of DkERS1 (Pang et al., 2007). In contrast, expression of FaETR2 was high in the flowers then decreased to a minimum in the small green fruits. During the subsequent fruit growth, it increased about 3-times to reach a maximum in the white fruits. Later on, although a slight decrease was observed, the expression remained high in the red fruits at well over twice that of the small green fruits. (Trainotti et al. 2005).

RhETR2 was expressed in all tissues: petals, floral buds, and leaves of F1 plants of ‘Vanilla’ and ‘Lavender’. The expression patterns of RhETR2 revealed some interesting results, in all F1

genotypes studied, RhETR2 produced the highest expression in petals and then in leaves and the lowest expression was in floral buds. However reverse Northern dot plot showed almost no differences between the expressions of RhETR2 between F1 genotypes, (Nr. 48 and Nr. 74) in petals, which agreed with RT-PCR results.

Moderate expression of RhETR3 was detected in carpels, petals, floral buds and leaves of 'Vanilla'. While in 'Lavender', it was very low in carpels and petals and undetectable in floral buds and leaves. It is also found that expression of RhETR3 differ with stage of development in 'Vanilla' Implying a possible role in flower development regulation, while in Lavender it was very low and constitutive regardless the growth stage.

Reverse Northern dot blot approved these differences in expression of RhETR3 particularly in petals, and to less extent in floral buds. These differences in RhETR3 Expression between 'Vanilla' and 'Lavender' suggested a possible role in ethylene sensitivity differences between both cultivars and therefore we further tried to explore our investigation to RhETR3 to find SNPs marker between both cultivars since our results showed always differences in RhETR3 expression between 'vanilla' and 'Lavender'. Previous findings of Müller et al. (2000b) are irreconcilable to our results; they reported that the level of mRNA for the ethylene receptor RhETR3 in petals of 'Bronze' was clearly higher than in 'Vanilla'. While RhETR3 was constitutively expressed at a very low level in 'Vanilla' and the expression of RhETR3 transcript increased with the beginning of senescence stage of 'Bronze' flowers. Expression of RhETR3 in cultivar 'Samantha' was at a level comparable to expression of RhETR1 in the petals of control flowers during the opening process, and was substantially enhanced by ethylene, but was not inhibited by STS, while in cultivar 'Kardinal', RhETR3 expression was temporarily promoted by ethylene immediately after the treatment; but was not altered by STS. (Tan et al., 2006), they found also that without ethylene treatment, the expression level of ethylene receptors was higher in 'Samantha' than in 'Kardinal'; the expression

of Rh-ETR3 was most readily enhanced by ethylene. They suggested that, RhETR3 might have the same substantial role of LeETR4 in tomato in responding to ethylene treatment in cut rose as RhETR3 shared the highest identity with LeETR4 (70%) among the other receptor genes. Ma et al. (2006) pointed out that the expression level of RhETR3 was always higher than RhETR1 that of in the same experiment, they indicated also that RhETR1 and RhETR3 transcripts were markedly strengthened by ethylene and weakened by 1-MCP, Buanong et al. (2005) found different results when she investigated RhETR3 expression in petals and leaves of both ‘Vanilla' and ‘Lavender' cultivars, she reported that, without any treatment, no expression of RhETR3 was detected in petals of both cultivars whereas, weak expression was detected in leaves of both of them. In F1 plants, RhETR3 was expressed mainly in petals regardless of the ethylene sensitivity of the plants tested, while in leaves and floral buds the amount or RhETR3 transcript was too low as RT-PCR results showed. When comparing F1 plant Nr. 84 and plant Nr. 74 by reverse Northern blot, we found high expression of RhETR3 in petals of both of them with slightly more tendency to hybridize in case of genotype Nr. 74 which is high sensitive to ethylene, even so this effect confirms the above indicated RT-PCR results.

The last ethylene receptor that was investigated is RhETR4 which was very low or not expressed in both of ‘Lavender’ and 'Vanilla' in all tissues and stages of flower development as obtained from RT-PCR results. Reverse Northern dot blot showed that higher expression of RhETR4 in petals of ‘Vanilla’ more than in ‘Lavender’, while in whole floral buds it was expressed the same in both cultivars. RT-PCR results of Buanong et al. (2005) revealed that RhETR4 was not expressed in petals of ‘vanilla’ and ‘Lavender’ but in only in leaves of ‘Vanilla’, these results are in agreement with what we found. In F1 plants, RhETR4 was not or very low expressed in the investigated tissues: petals, floral buds and in leaves. These results support the pervious results obtained from the parents (‘Vanilla’ and ‘Lavender’). Reverse Northern blot of F1 plants, showed high expression

difference between plants Nr. 48 and plant Nr. 74. The ethylene low sensitive plants (Nr. 48) showed higher expression.

In the study of Müller et al. (2000b), RhETR4 was isolated along with RhETR2 and RhETR3, the authors investigated the expression of RhETR2 and RhETR3 but never mentioned any expression details of RhETR4, they reported that the deduced amino acid sequence of a 547 bp internal fragment of RhETR4 is 79% identical to that for RhETR1 and 74% identical to hat for AtERS1.

Our results are consistent with reduction in the expression level of LeETR4 that leads to enhance ethylene responses in tomato plants, over expression of NR can compensate for the loss of LeETR4 and eliminates ethylene sensitivity. These results reveal that mechanisms of ethylene perception are likely conserved among flowering plants (Tieman et al., 2000).

Yau et al. (2004) findings as they reported that expression levels of both OS-ETR3 and OS-*ETR4* are too low to be detected by conventional RNA gel blot analysis. Thus RT-PCR was performed to measure their mRNA levels. Results showed that both OS-ETR3 and OS-ETR4 mRNAs were present in young green rice seedlings and anthers.

Ethylene receptors have been shown to act as negative regulators of ethylene responses in plants (Hua and Meyerowitz, 1998; Tieman et al., 2000). In this mechanism, the ethylene receptors have two states. When ethylene is absent and does not bind to its receptors, the receptors are active and consequently there is no ethylene response in the plants. When ethylene is present and binds to its receptors, the receptors are inactive. This inactive state will lead to the ethylene responses (Hua and Meyerowitz, 1998). This mechanism of negative regulation indicates an inverse relationship between the level of ethylene receptors and the sensitivity to ethylene. Actually, in a transgenic tomato plant, reduced expression of an ethylene receptor gene, LeETR4, resulted in constitutive ethylene responses such as leaf epinasty and accelerated flower senescence (Tieman et al., 2000). By contrast, Muller et al. recently reported a parallel relationship between the level of ethylene

receptor transcripts and the flower longevity, which is regulated by sensitivity to ethylene in rose flowers (Muller et al., 2000). They found that the expression of RhETR3, one of four genes identified in rose, increased in senescing flowers of 'Bronze', a cultivar with a short floral life, while it remained at low levels in 'Vanilla', a cultivar with a long floral life. Furthermore, they found that the expression of the gene was increased by ABA and ethylene treatment, which stimulated the senescence of the rose flowers. From these results, they proposed that the differences in flower life among rose cultivars could be due to differences in receptor levels.

4.2.2.2 Expression of ACSs

Ethylene biosynthesis enzymes genes are differently expresses in Vanilla and in Lavender, the expression was always higher in Vanilla than in Lavender, except in Lavender pistil for RhACS1 in which it was higher than in the same tissue in 'Vanilla'. RhACS2 is highly expressed in 'Vanilla' and in 'Lavender'.

In higher plants, ethylene is biosynthesis have been well documented in which 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase catalyze the reactions from S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid ACC by ACC synthase (ACS) and from ACC to ethylene by ACC oxidase (Yang and Hoffman, 1984). In this study five ACC synthase genes were studied, RhACS1 isolated by Wang et. al., (2004) and the other four RhACS2-4 were isolated by Mibus and Serek (2004). The RT-PCR result indicated that only *RhACS1* and *RhACS2* were expressed in both vanilla and Lavender. These results are in agree with Liu et. al. 2013. As they reported an increase in level expression of *RhACS1* and *RhACS2* in cut roses throughout the dehydration period in which more endogenous ethylene were produced. They reported also that expression levels of *RhACS3* remained constant however the mRNA levels of the *RhACS4* and *RhACS5* genes were hardly detectable, these findings are fully supported our

results. In floral buds, carpels, and pistils, the expression of *RhACS1-2* was higher than in Leaves. Expression of both *RhACS1-2* increased with development of flower till senescence. The F1 expression results suggested that the *RhACS1* and *RhACS2* are more expressed in floral buds of high ethylene sensitive plants that in the vegetative leaves of low ethylene sensitive plants. Recent study of Khan et. al. (2015) found that the promoter activities of *RhACS1* and *RhACS2* were strong during the development from young seedlings to mature flowering plants in various organs, including hypocotyls, cotyledons, leaves, roots and lateral roots.

Meng et al. (2014) assumed that Accumulation of RhACS1 and RhACS2 proteins may be responsible for rapid ethylene production in rose gynoecia during rehydration. The expression ACS8 in Arabidopsis which is controlled by light, by the circadian clock, and by negative feedback regulation through ethylene signaling are strongly correlated Ethylene production levels (Thain et al. 2004). From our results, we can conclude that the expression of RhACS1 and RhACS2, mostly contribute to ethylene production in floral organs bud, petal and carpels than in leaves. These results are in agree with our findings from reverse northern dot hybridization. Many studies have indicated that ACS gene expression can be negatively regulated by ethylene in vegetative tissues such as wounded winter squash tissue (Nakajima et al., 1990), pea seedlings (Peck and Kende, 1998) and pre-climacteric fruit such as tomato (Barry et al., 2000).

4.2.2.3 Expression of CTR1-2 and EIN3, EIL

Many studies have defined a number of genes involved in the signal transduction pathway of ethylene. *RhCTR1*, *RhCTR2*, *RhEIN3*, and *RhEIL* transcripts were found in all tissues investigated of both ‘Vanilla’ and ‘Lavender’. Expression of *RhCTR1* in the carpels of ‘Vanilla’ was higher than in ‘Lavender’ in the same tissue. On the contrary with Müller et. al. (2002) results, in which they indicated that *RhCTR1* expression increased during flower senescence, we found that expression of *RhCTR1* was strong in the early stages and decreased gradually when the flower

become older. This agreed with the findings in carnation, in which the level of EIL1 (EIN3-like) transcript decreased in flower petals during natural senescence or senescence stimulated by exogenous ethylene or ABA (Waki et al., 2001). Buanong et al. (2005) also confirmed the expression of in control treatments in petals. Expression of *RhCTR1, RhCTR2,* RhEIN3, *and RhEIL* showed differences between Vanilla and Lavender, for example RhEIN3 was expressed more in vanilla than in lavender in carpels, petals, and floral buds, whereas in leaves show no differences between both varieties. It is interesting that the expression of RhEIN3 was higher in early stages in both varsities, suggesting that would play a role in sensitivity to ethylene and require further investigations. Tan et al. (2006) investigated the expression of CTRs and EIN3 genes in curt roses, they reported that there is no clear effect of ethylene was found on the expression of CTRs and EIN3 genes. But in control flower the expression patterns were different and were stronger in cv. Samantha than in cv. Kardinal during the flower opening. The expression studies of *RhCTR1, RhCTR2*, RhEIN3, *and RhEIL* in In F1 plants confirmed the obtained results from parents and showed more obvious findings that low ethylene sensitive plant exhibited stronger expression that the high ethylene sensitive plants. Reverse northern dot blot hybridization confirmed also these results.

A lack of clear correlation between transcription of ethylene signal transduction genes and ethylene sensitivity was also noted by Ahmadi et al. (2009), but it was established for more genes in this study. These observations indicate that much of the variation seen for transcript levels may be due to random factors (such as variation in the genotypes and epigenetic state of the analyzed plants) that are not important for ethylene sensitivity, and that other genes or factors could more tightly regulate the sensitivity to ethylene. In accordance with this, recent molecular investigations of the ethylene signal transduction pathway have revealed other processes, such as proteasomal

degradation and MAP kinase cascades, that are involved in the signal processes (Cho and Yoo 2015).

4.3 Identification of candidate SNPs markers associated with ethylene sensitivity with CAPS

The development of molecular markers is very useful for plant breeders to identify cultivars with special characteristics. SNPs are single base pair positions in genomic DNA at which different sequences alternatives (alleles) exist in normal individuals in some population(s), wherein at least frequent allele has an abundance of 1% or greater. Thus, single insertion/deletion variants would not formally be considered to be SNPs. In principle, SNPs could be bi-, tri-, or tetra-allelic polymorphisms. However, tri-allelic and tetra-allelic SNPs are rare almost to the point of non-existence in Humans, and so SNPs are sometimes simply referred to as bi-allelic or di-allelic markers. This is somewhat misleading because SNPs are only a subset of all possible bi-allelic polymorphisms (Brookes, 1999). Moreover, SNPs leading to a non-conservative amino acid exchange may be connected with a phenotypic effect, which enable the development of reverse genetic screens based on chemical mutagenesis (McCallum et al. 2000). Cleaved amplified polymorphic sequence (CAPS) is defined as a combination of the PCR and RFLP, and it was first called PCR-RFLP (Maeda et al., 1990). The method includes amplification of a target DNA through PCR, then by digesting the PCR product with restriction enzymes (Jarvis et al., 1994). In this study, CAPS method will offer a more reliable and rapid method to identify ethylene sensitive seedlings than the usual test with exogenous ethylene application method.

In order to prove the candidate SNPs that found in EhETR3 and RhEIN3, eight plants from the F1 generation were examined. The digestion of PCR products was exactly as expected proving that it

as valid SNPs. Although CAPS method was successful and several SNPs marker was detected, there were no relationship between the SNPs and ethylene sensitivity. This means that the detected SNPs markers weren't closely linked to high or low sensitivity of ethylene in the studied plants. The detected SNPs failed to distinguish sensitivity to ethylene between the tested plants, either between parent (Vanilla and Lavender), as well as between the F1 plants. It is suggested that more plants should be tested prove the SNPS. Other explanation, that the investigated genes may not related directly to the ethylene sensitivity and other genes are might be responsible for the variation the ethylene sensitivity. Much more study is need to understand the structure of rose genome and its complexity as the majority of current roses, including the miniature potted roses (*Rosa x hybrida L.*) have been produced through traditional breeding programs and among thousands of existing varieties and cultivars. In the genus *Rosa,* chromosome numbers are multiples of seven and range from (2n=2x=14) to (2n=8x=56) but most species are diploid or tetraploid (Rout et al. 1999; De Cock et al., 2007), modern roses are sorted into several classes include Polyanthas (2n=2x), Hybrid Teas (2n=3x, 4x), Floribundas (2n=3x, 4x) and Miniatures (2n=2x, 3x or 4x) (Yokoya et al., 2000),

CHAPTER 5

CONCLUSION & OUTLOOK

5. CONCLUSION AND OUTLOOK

From the observations in the present work it is not possible to find a clear correlation between transcript levels of the analyzed genes and the ethylene-sensitivity of the studied rose cultivars. A lack of clear correlation between transcription of ethylene signal transduction genes and ethylene sensitivity was also noted by Ahmadi et al. (2009), but it was established for more genes in this study. These observations indicate that much of the variation seen for transcript levels may be due to random factors (such as variation in the genotypes and epigenetic state of the analyzed plants) that are not important for ethylene sensitivity, and that other genes or factors could more tightly regulate the sensitivity to ethylene. In accordance with this, recent molecular investigations of the ethylene signal transduction pathway have revealed other processes, such as proteasomal degradation and MAP kinase cascades, that are involved in the signal processes (Cho and Yoo 2015). It is therefore likely that transcription of other genes involved in ethylene signal transduction, posttranscriptional or posttranslational control, or crosstalk with other signal transduction pathways may influence the degree of ethylene sensitivity of miniature roses.

However, some genes that showed markedly differences in gene expression were further studied to find some genetic markers between Vanilla and Lavender such as RhETR3 and RhEIN3, such genes could play an important role in postharvest life of miniature roses and should be studied more in details in future to develop SNP markers of ethylene sensitivity in miniature roses.

This research provides three validated SNPs in partial DNA sequence of the RhETR3 alleles analyzed and two SNPs marker in RhEIN3. These new CAPS markers might be important for the quick and cost-effective genetic characterization of diverse miniature potted roses germplasm and probably to be utilized for marker assisted selections studies in roses. More research is needed to establish clear molecular differences between the ethylene low and high sensitive plants, and thus

to provide genetic markers to distinguish between these cultivars. Other ethylene related genes should be also studies in order to explain the differences in postharvest life of miniature potted roses, For example, 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) genes and ethylene response factors (ERFs).

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APPENDICES

Appendices: paper 1. Expression analysis by RT-PCR of genes involved in ethylene synthesis and signal transduction in miniature roses

APPENDICES

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Expression analysis by RT-PCR of genes involved in ethylene synthesis and signal transduction in miniature roses

Mohammad Al-Salem, Margrethe Serek *

Leibniz University of Hannover, Faculty of Natural Sciences, Institute of Horticulture Production Systems, Floriculture, Herrenhaeuser Str. 2, D-30419 Hanover, Germany

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ABSTRACT

Ethylene is one of many factors that affect the quality, appearance and longevity of miniature roses. In this study RT-PCR is used to compare the expression of ethylene biosynthetic genes and ethylene signal transduction genes at different stages of flower development in the two cultivars 'Vanilla' and 'Lavender', which show low and high ethylene-sensitivity, respectively, and their F1 offspring. The genes for the ethylene receptors RhETR1, RhETR2 and RhETR3, the genes for the receptor-associated signaling proteins RhCTR1 and RhCTR2, the genes for the transcription factors RhEIN3 and RhEIL, and the genes for the ACC synthases RhACS1 and RhACS2 each had an expression pattern that varied between the tested plants and tissues, but could not be correlated with the ethylene sensitivity of the plants. *RhETR1, RhETR2, RhETR3* and *RhEIN3* were, e.g., expressed more in 'Vanilla' than in 'Lavender' in most, but not all, of the investigated tissues, but were in general not expressed more in progeny with low sensitivity than in progeny with high sensitivity. No expression was detected for the 4 other genes that were investigated, i.e. genes for RhETR4, RhACS3, RhACS4 and RhACS5. It is concluded that the precise transcriptional activities of the tested genes do not appear to be crucial in determining the ethylene sensitivity of miniature roses. It therefore appears likely that transcription of other genes involved in ethylene signal transduction, posttranscriptional or posttranslational control, or crosstalk with other signal transduction pathways may be important for the degree of ethylene sensitivity of miniature roses.

1. Introduction

Miniature rose, *Rosa hybrida* L., is one of the most economically important ornamental plants for indoor use. Miniature potted roses have become very popular in the last two decades due to their availability in a wide range of colors, sizes, growth habits, and to improved postharvest performance. The annual world production is estimated to be more than 100 million plants, and Denmark is the largest producer with nearly 50% of the world's production (Pemberton et al., 2003). The number of miniature rose plants sold in 2015 at Flora Holland, the world largest flower auction, reached 47 million, generating revenues of EUR 57 million (FloraHolland, 2016).

Ethylene is one of the most important factors affecting the quality, appearance and longevity of many ornamentals (Serek et al., 2006; Ferrante et al., 2015). In miniature roses, ethylene can cause unwanted effects such as premature and accelerated wilting and abscission of leaves, floral buds, petals and flowers, as well as leaf yellowing or discoloration (Serek 1993; Andersen et al., 2004). The display life of some commercially grown miniature potted rose cultivars was compared by Müller et al. (1998), and it was found that it varied considerably, at least partly due to differences in endogenous ethylene production and sensitivity to exogenous ethylene.

In higher plants, the mechanism of ethylene biosynthesis has been well investigated (Yang and Hoffman, 1984; Xu and Zhang 2015). Ethylene signal transduction has also been investigated extensively, resulting in a model encompassing the following well-established aspects (Merchante et al., 2013; Cho and Yoo 2015; Gallie 2015; Shakeel et al., 2015). In the absence of ethylene, the ethylene receptors, located in the ER membrane, activate CTR1 (and its homologs) which is associated with the receptors and, when activated, phosphorylates and inhibits EIN2 (a membrane protein also located in the ER membrane). In the presence of ethylene, CTR1 is "switched off", and EIN2 becomes dephosphorylated, leading to release of its C-terminal domain (Ju et al., 2012). This domain translocates to the nucleus, where it blocks degradation of the transcription factors EIN3/EIL1 and thus activates the transcriptional response to ethylene. Other, less well-characterized proteins,

* Corresponding author.
E-mail address: serek@zier.uni-hannover.de (M. Serek).

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Fig. 1. Three flower stages for 'Vanilla' (left) and 'Lavender' (right): 1 Flowers in the opening phase. 2 Flowers fully open. 3 Flowers in initial phase of senescence.

an internal control for normalization of the other gene expressions. Corrections for differences in amplicon lengths for *Rh-ß-actin* and the other investigated transcripts were included in the normalizations. The exponential range was determined by carrying out PCR for 20–45 cycles using serial dilution of a fixed quantity of *Rh-ß-actin* cDNA (Supplementary Fig. S1). As seen, the reaction appears to be in the exponential range for 30 or fewer cycles. Therefore, the RT-PCR reactions in this work was run for 30 cycles and with dilutions containing about as much *Rh-ß-actin* cDNA as the 10 fold diluted samples used in Supplementary Fig. S1.

2.4. RT-PCR

Total RNA was reverse transcribed to cDNA as described above and PCR reactions were performed on the cDNA in a T3 thermocycler (Biometra, Germany) under the following conditions: an initial denaturation step at 95 °C for 2 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 50–62 °C for 40 s, and extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were electrophoresed in a 1% agarose gel with 0.3 μg mL^{-1} ethidium bromide in 1 × TAE buffer and visualized using a BioDoc-Analyze UV transilluminator (Biometra, Germany). As a negative control for RT-PCR, a mixture of different randomly chosen RNA samples were subjected to the same reaction without reverse transcriptase. All RT-PCR experiments were repeated three times for each total RNA preparation.

2.5. Densitometric analysis of RT-PCR results

Negative images of the agarose gels were analyzed to quantity the optical density of PCR bands from each sample using ImageQuant software (Amersham, GE Healthcare UK limited, Buckinghamshire, UK). The results were expressed as the relative density in comparison to the optical density of the background and normalized to the expression of the housekeeping gene for ß-actin, which is assumed to be equally expressed in most cells.

3. Results

3.1. Ethylene receptor genes (RhETR1-4)

RhETR1, *RhETR2* and *RhETR3* transcripts were detected in all tissues tested for both 'Vanilla' and 'Lavender' (Fig. 2). Expression of *RhETR4* was not detected, regardless of the type of tissue or growth stage (data not shown). In carpels, the amount of *RhETR1* mRNA in 'Vanilla' was higher than for 'Lavender' at all developmental stages (Fig. 2). In petals, the amount of *RhETR2* mRNA was higher for 'Vanilla' than for 'Lavender' at stage 2 and 3 and all three transcripts reached their maximum at stage 3 for 'Vanilla' but stage 2 for 'Lavender'. In floral buds and in leaves the level of all three receptor transcripts was higher for 'Vanilla' than for 'Lavender'. *RhETR3* mRNA was higher for 'Vanilla' in buds and leaves whereas it was not expressed in Lavender in the same tissues (Fig. 2). The total amount of transcript for the three receptors (estimated as the sum of the column heights) was higher for 'Vanilla' than for 'Lavender' in all cases except for stage 1 petals.

For the selected F1 genotypes (Section 2.1), expression of *RhETR1-4* was measured in petals of fully blooming flowers (stage 2, Fig. 1), floral buds and leaves from each of the selected F1 genotypes. As for the parents, no expression of *RhETR4* was detected in the investigated tissues. In all cases except for genotype 48 buds the dominant transcripts are *RhETR2* (Supplementary Fig. S2). In general, the differences between the genotypes of low and high ethylene sensitivity are small, and less conspicuous than some of the differences seen between 'Vanilla' and 'Lavender'.

3.2. Genes for the signal transduction proteins RhCTR1 and RhCTR2

Expression of the *RhCTR1* and *RhCTR2* genes, which encode homologous protein kinases involved in ethylene signal transduction, were analyzed in 'Vanilla' and 'Lavender' (Fig. 3). *RhCTR2* was significantly more expressed than *RhCTR1* in all investigated tissues, except for 'Lavender' stage 3 carpels where both showed very low expression. The total amount of transcript for the two genes (estimated as the sum of the column heights) varied, with the exception just mentioned, from 0.33 (for 'Vanilla' stage 3 carpels) and 0.97 (for 'Vanilla' stage 3 petals). It was relatively constant for all 6 samples of petals.

For the selected F1 plants the expression of *RhCTR1* was significantly higher than, and that of *RhCTR2* was, in general, at the same level as, the expressions found in 'Vanilla' and 'Lavender' (Supplementary Fig. S3). Accordingly, the sum of the expressions was higher than for the parent plants.

3.3. Genes for the transcription factors RhEIN3 and RhEIL

Expression of the *RhEIN3* and *RhEIL* genes, which encode homologous transcription factors involved in the ethylene signal pathway, were detected in all investigated tissues from 'Vanilla' and 'Lavender' (Fig. 4). In all cases, except for 'Vanilla' stage 1 carpels and 'Lavender' stage 3 petals, buds and leaves, the expression of *RhEIL* exceeded that of *RhEIN3*. In petals, the amount of both transcripts was maximal at stage 2 and 3 for 'Vanilla' but at stage 1 and 2 for 'Lavender'.

As for the parent plants, expression analysis of stage 2 petals, buds and leaves from the selected F1 plants detected both transcripts (Supplementary Fig. S4) and more *RhEIL* than *RhEIN3* transcript. There is only little variation between expression in the 4 genotypes. For the petals, the total expressions are similar to that of stage 2 petals from the parent plants, and for leaves the total expressions are also similar.

3.4. Ethylene biosynthetic genes (RhACS1-5)

Expression of the ethylene biosynthetic enzyme genes *RhACS1-5* was analyzed in 'Vanilla' and 'Lavender'. Whereas *RhACS1* and *RhACS2* transcripts were detected in all investigated tissues for both cultivars (Fig. 5), *RhACS3-5* transcripts were not detected in any of

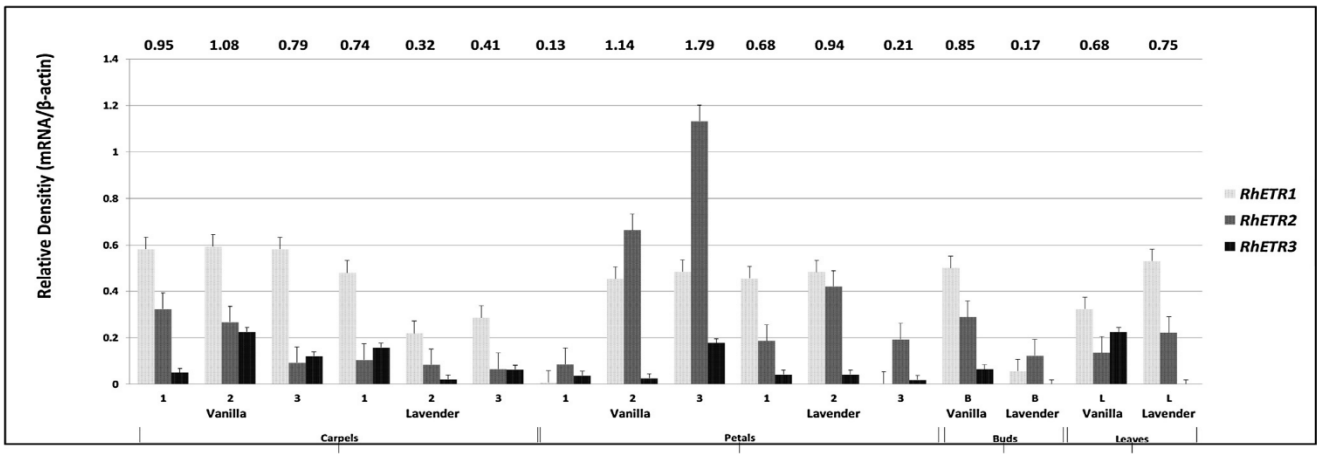

Fig. 2. Expression patterns for *RhETR1, RhETR2 and RhETR3* at three developmental stages of (carpels and petals), and in floral buds and leaves of 'Vanilla' and 'Lavender' as determined by RT–PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard *Rhβ-actin*, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT–PCR reactions; error bars represent standard error of the mean. The numbers at the top are the sum of the heights of the columns for *RhETR1–3*.

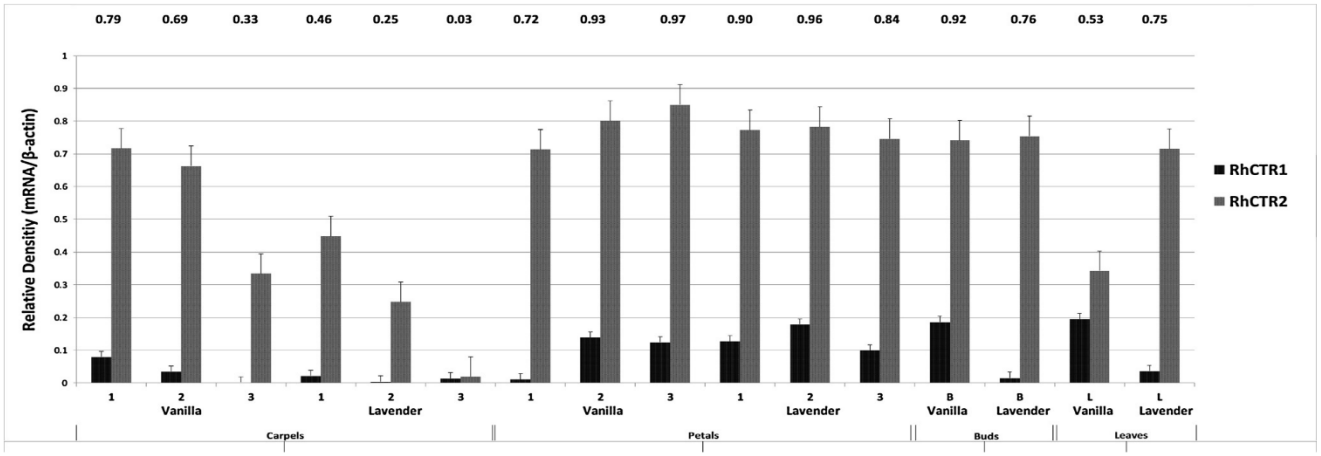

Fig. 3. Expression patterns for *RhCTR1* and *RhCTR2* at three developmental stages of (carpels and petals), and in floral buds and leaves of 'Vanilla' and 'Lavender' as determined by RT–PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard *Rhβ-actin*, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT–PCR reactions; error bars represent standard error of the mean. The numbers at the top are the sum of the heights of the columns for *RhCTR1* and *RhCTR2*.

the investigated tissues (data not shown). In 'Vanilla' and 'Lavender' carpels and in 'Vanilla' petals, buds and leaves *RhACS2* was more expressed than *RhACS1*, whereas the opposite was the case for 'Lavender' petals (except for stage 1), buds and leaves. The sum of the two transcript levels is in all cases larger for 'Vanilla' than for 'Lavender'.

As for the parent plants, expression analysis of stage 2 petals, buds and leaves from the selected F1 plants detected *RhACS1* and *RhACS2* transcripts (Supplementary Fig. S5), but no *RhACS3*, *RhACS4* and *RhACS5* transcripts (data not shown), in the investigated tissues. The expression of *RhACS1* was always higher than the *RhACS2* expression in petal tissue, as for 'Lavender' but not for 'Vanilla' (Fig. 5), regardless of sensitivity to ethylene (Supplementary Fig. S5). In addition, the sum of the two transcript levels in petals is larger than for both 'Lavender' and 'Vanilla' for all 4 genotypes. For leaves and buds the patterns were more variable, but without conspicuous correlation with ethylene sensitivity.

4. Discussion

To clarify the reasons for the significant differences in postharvest characteristics of miniature potted roses, mRNA expression patterns of genes involved in ethylene biosynthesis, perception and signal transduction pathways were investigated and compared for the cultivars 'Vanilla' and 'Lavender', which show low and high ethylene sensitivity, respectively, and plants selected among their F1 offspring for low and high ethylene sensitivity (Section 2.1).

Based on the current model of ethylene signal transduction (briefly described in the Introduction), and the experimental evidence behind it, it may be expected that plants with increased levels of receptor protein, increased CTR1 levels, decreased EIN2 levels, or decreased EIN3/EIL levels should show decreased ethylene sensitivity. We investigated the transcript levels for the cloned *Rosa hybrida* genes for ethylene signal transduction proteins in the above-mentioned cultivars to look for a possible correlation

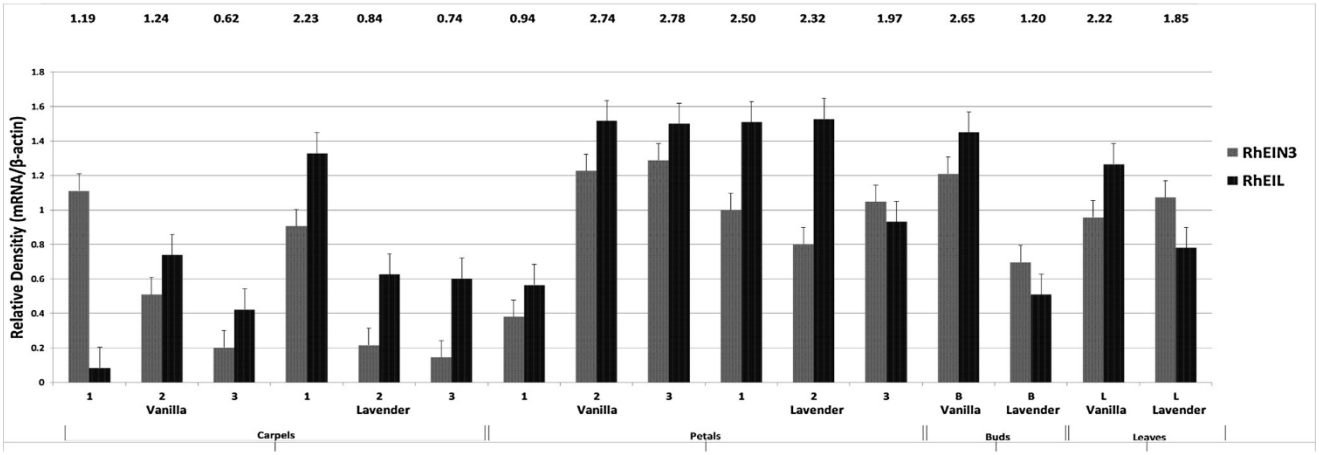

Fig. 4. Expression patterns for *RhEIN3* and *RhEIL* at three developmental stages of (carpels and petals), and in floral buds and leaves of 'Vanilla' and 'Lavender' as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard *Rhβ-actin*, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions; error bars represent standard error of the mean. The numbers at the top are the sum of the heights of the columns for *RhEIN3* and *RhEIL*.

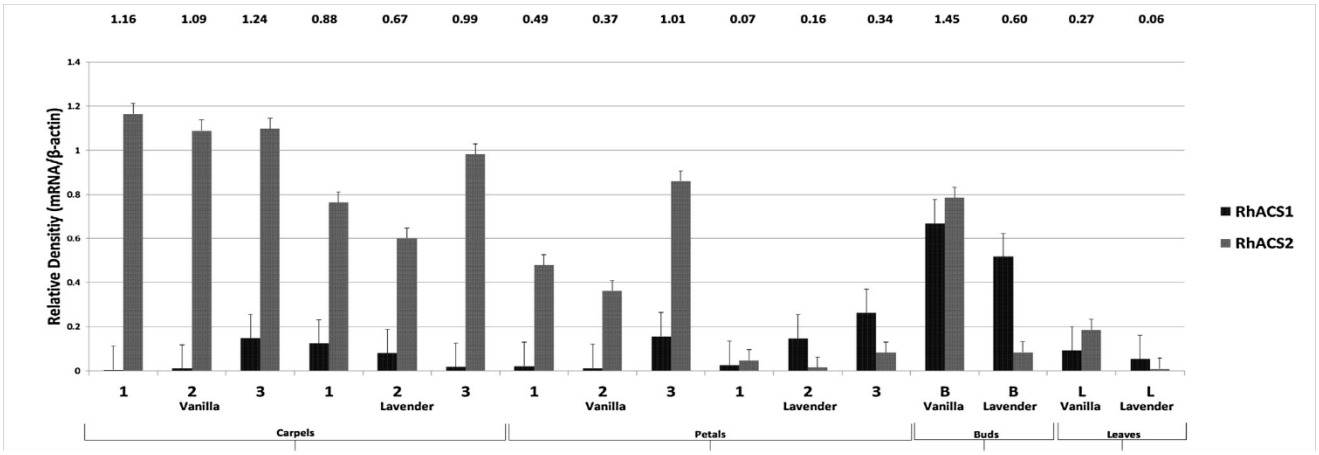

Fig. 5. Expression patterns for *RhACS1 and RhACS2* at three developmental stages of (carpels and petals), and in floral buds and leaves of 'Vanilla' and 'Lavender' as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard *Rhβ-actin*, with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions; error bars represent standard error of the mean. The numbers at the top are the sum of the heights of the columns for *RhACS1* and *RhACS2*.

between transcript levels and ethylene sensitivity that could confirm (or disagree with) these expectations. *RhEIN2* is not yet cloned and was therefore not investigated.

Müller et al. (2000a) reported that the expression of *RhETR1* in petals from the cultivar 'Bronze' with short flower life was clearly higher than for the long-lasting 'Vanilla', in contrast to what could be expected from the ethylene signal transduction model. We found, however, that the amount of *RhETR1* mRNA in 'Vanilla' was higher than for the short-lasting cultivar 'Lavender' at all developmental stages in carpels, at stage 3 in petals, and in buds (Fig. 2). In floral buds and in leaves the levels of all three receptor transcripts were higher for 'Vanilla' than for 'Lavender' (Fig. 2). In addition, the total amount of transcript for RhETR1-3 was higher for 'Vanilla' than for 'Lavender' in all cases except for stage 1 petals and leaves. These findings are what would be expected from the signal transduction pathway. The expression of *RhETR1* in F1 progeny plants of 'Vanilla' and 'Lavender' was evaluated for petals, floral buds, and leaves (Supplementary Fig. S2). *RhETR1* was expressed in all tissues, and no clear differences were seen between the long-lasting genotypes 48 and 131 and the short-lasting genotypes 50 and 74. The long-lasting genotypes, however, had significantly lower total receptor expression in buds and leaves (but not in petals) than the short-lasting genotypes, which is not what could be expected from the model of ethylene signal transduction. For 'Vanilla', in contrast, the total receptor expression in buds is larger than for 'Lavender' buds (Fig. 2).

In petals, the amount of *RhETR2* mRNA was higher for 'Vanilla' than for 'Lavender' at stage 2 and 3 and all three transcripts reached their maximum at stage 3 for 'Vanilla' but stage 2 for 'Lavender' (Fig. 2). *RhETR2* expression was higher than *RhETR1* expression for 'Vanilla' petals, 'Lavender' stage 3 petals and 'Lavender' buds, whereas the opposite was the case for the other analyzed tissues (Fig. 2). These results for petal *RhETR2* transcripts are not in agreement with observations by Müller et al. (2000b), who found that

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2016.12.029.

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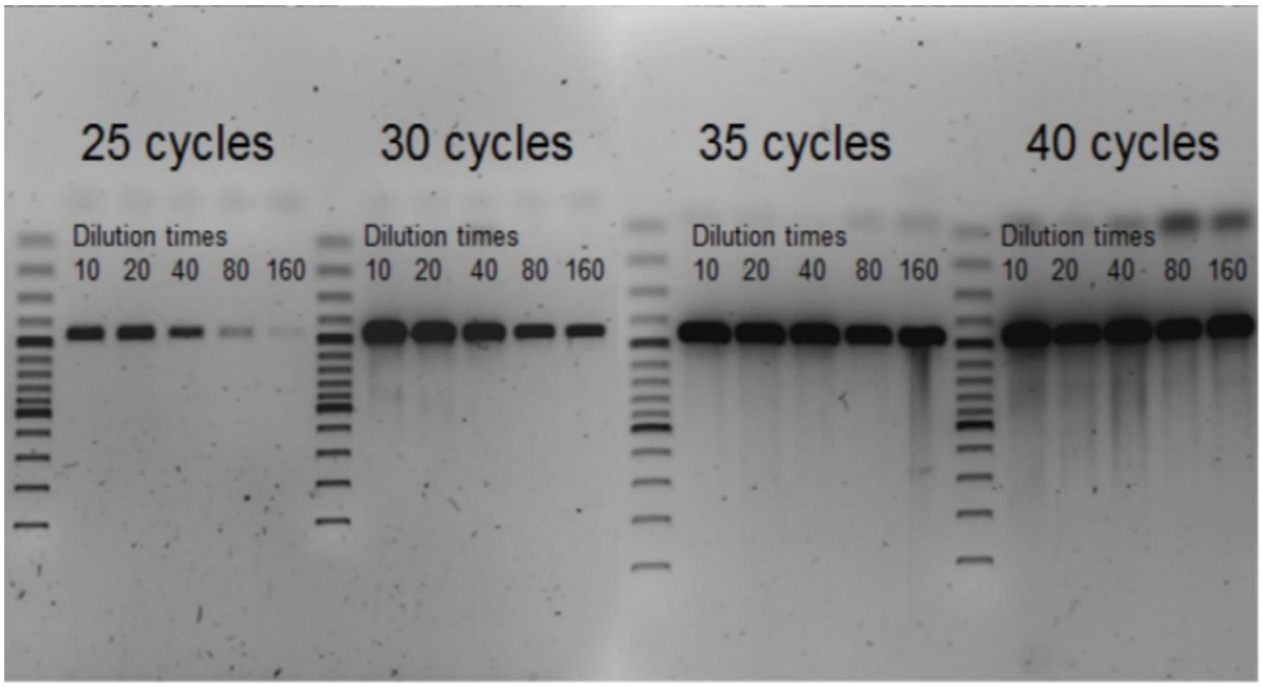

Figure S1. RT-PCR with *Rh-β-actin* primers performed with 25-40 cycles and serial dilution of a fixed quantity of *Rh-ß-actin* cDNA. The reaction appears to be in the exponential range for 30 or fewer cycles.

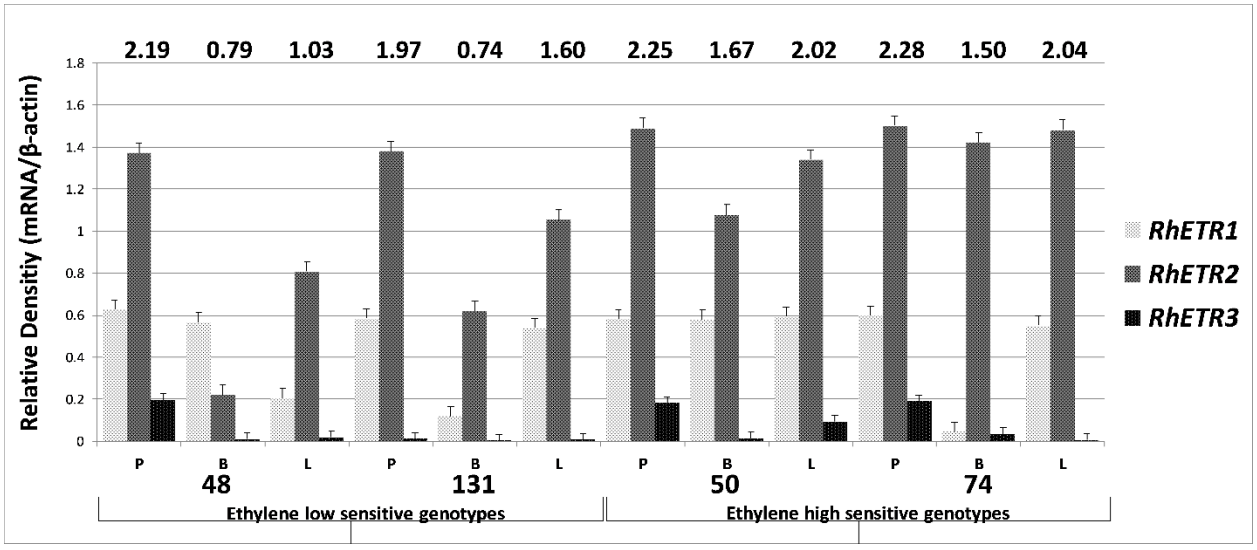

Figure S2. Expression patterns for *RhETR1*, *RhETR2* and *RhETR3* of F1 plants in petals (P), floral buds (B) and leaves (L) as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard *Rhβ-actin,* with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions; error bars represent standard error of the mean**.** The numbers at the top are the sum of the heights of the columns for *RhETR1-3*.

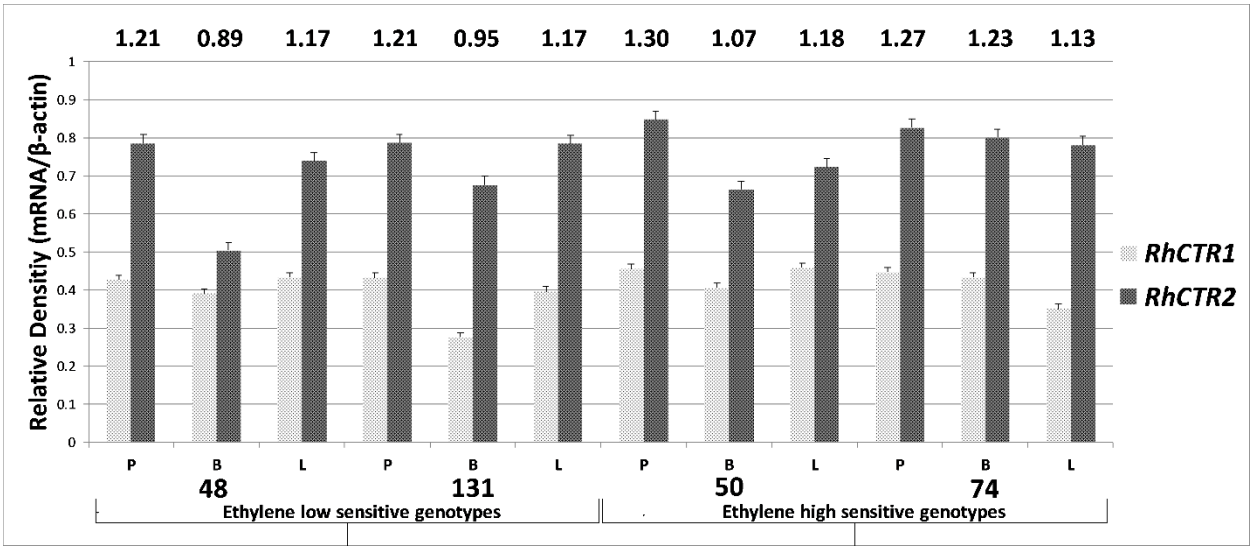

Figure S3. Expression patterns for *RhCTR1, RhCTR2* of F1 plants in petals (P), floral buds (B) and leaves (L) as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard *Rhβ-actin,* with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions; error bars represent standard error of the mean**.** The numbers at the top are the sum of the heights of the columns for *RhCTR1* and *RhCTR2*.

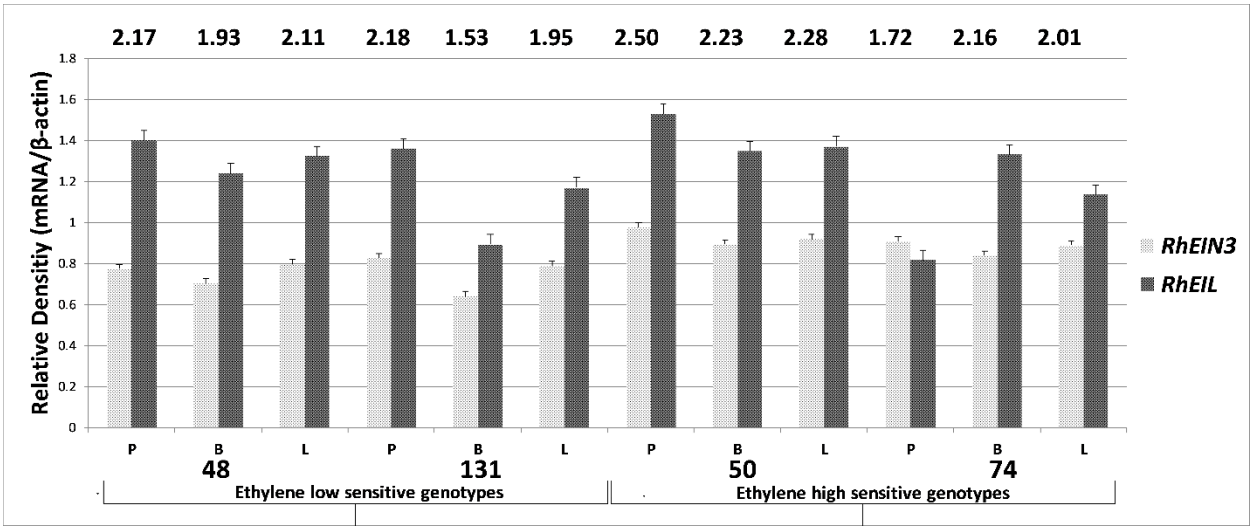

Figure S4. Expression patterns for *RhEIN3* and *RhEIL* of F1 plants in petals (P), floral buds (B) and leaves (L) as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard *Rhβ-actin,* with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions; error bars represent standard error of the mean**.** The numbers at the top are the sum of the heights of the columns for *RhEIN3* and *RhEIL*.

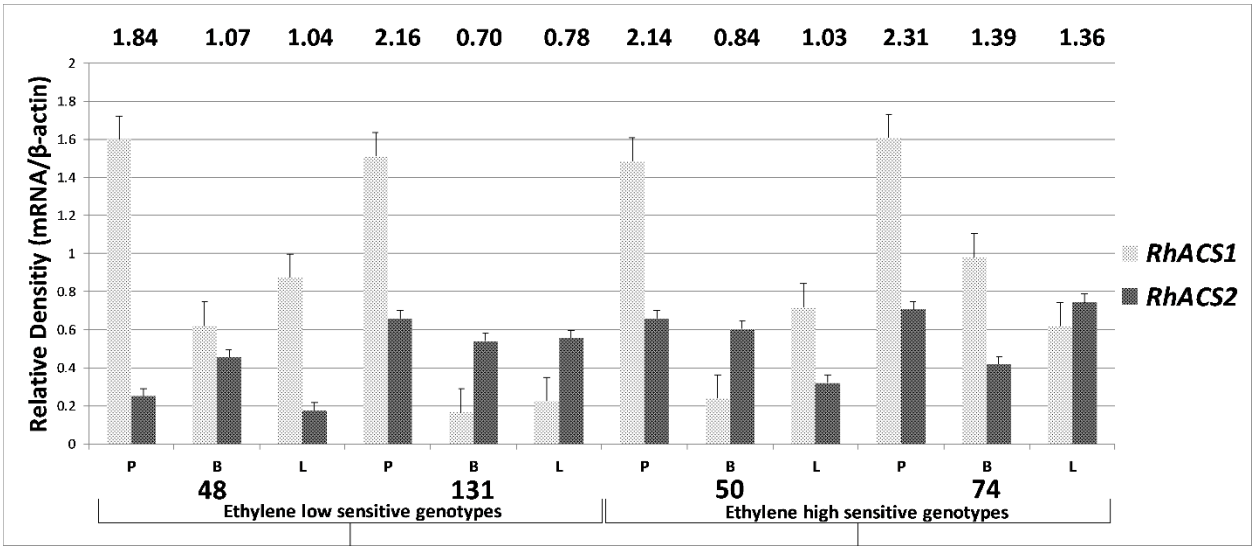

Figure S5. Expression patterns for *RhACS1 and RhACS2* of F1 plants in petals (P), floral buds (B) and leaves (L) as determined by RT-PCR. Each column represents the optical density of the PCR band divided by the optical density of the band for the internal standard *Rhβ-actin,* with correction for difference in amplicon sizes. The values are given as means of densitometric units of three RT-PCR reactions; error bars represent standard error of the mean**.** The numbers at the top are the sum of the heights of the columns for *RhACS1* and *RhACS2*.

Table S1. Primer pairs used for RT-PCR of the investigated genes.

Acc. No.	Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon size (bp)
BI977396	*Rh-ß-Actin*	CCATGTTCCCTGGTATTGCT	GCCTTTGCAATCCACATCTA	395
AF394914	*RhETR1*	TGGTATGAACCTTCAACTTTCTCA	CGCATAGACTCTTCAAGAATAGCA	393
AF127220	*RhETR2*	CTCAAACTTCCAAATCAATGACTG	ATATTCTGCTCCATTAGCAGATCC	213
AF154119	*RhETR3*	CACTGCTATAACGCTCATCACTCT	CATTAGTTGGGACTCTTCAAGGAT	661
AF159172	*RhETR4*	TTTGAATCTGCAACTTTCTCACAC	GCATTTCGTGGTTCATGACAG	500
AY032953	*RhCTR1*	GGCTCTGATGTTGCTGTGAA	TCAATGGCCTCAAAGATTCC	706
AY029067	*RhCTR2*	TTCCTTCCAAGGGGAAGTCT	CCCACTCCAAGCCAATTTTA	375
AF443783	*RhEIN3*	CCCTGCAGCCATAGACAAGT	ACCCTGATTTCATCCACCAA	236
AY052825	*RhEIL*	TCCCTGGTTTGATGGAAGAC	GAGGCCACCATTCCTCATTA	192
AY061946	*RhACS1*	AAACGTCACCGTTCCAACTC	CTGAATTTCCGATGGCCTTA	205
AY525066	*RhACS2*	AAAAACCCAGAAGCCTCCAT	AAGGAACGGGAACCAGAAAT	250
AY525067	*RhACS3*	CCATGGCCTTTTGTCCTTTA	GGGTTGGAGGGGTTTGTAAT	126
AY525068	*RhACS4*	GCTTCCAACTTGGGATCAAA	TGGGGTTGGAACTAGCAAAG	237
AY525069	*RhACS5*	CAGCCGGATTCAAGAGAAAC	GGCGAGGCAAAACATAAGAG	203

CURRICULUM VITAE (2017)

EMPLOYMENT SUMMARY:

Staff scientist & PhD candidate
Institute of Horticultural Production Systems, **10.2003-Present**
Floriculture Section
University of Hannover, Hannover, Germany.

German Intensive Course **04.2003-09.2003**
interDaF at the Herder Institute,
University of Leipzig.

Teaching & Research Assistant **01.2001-04.2003**
Plant Production Department,
Mutah University, Karak, Jordan

UNIVERSITY HIGHER EDUCATION & QUALIFICATIONS:

B.Sc., Plant Sciences, **10.1993-06.1997**
Jordan University of Science and Technology.
Irbid, Jordan.

M.Sc., Horticulture, **10.1997-04.2000**
Jordan University of Science and Technology,
Irbid, Jordan.
Thesis: Propagation of oriental strawberry tree (Arbutus andrachne L.). (Assistantship from Jordan University of Science and Technology).

PRIMARY & SECONDARY SCHOOL:

Primary school **1981-1991**
Rhaba primary school, Rhaba, Jordan.

LANGUAGES:

Arabic: Mother language
English: Excellent oral and written communication skills
German: very good

SCHOLARSHIPS AND AWARDS:

JUST Teaching Assistantship to get M.Sc. in Horticulture from Jordan University of Science and Technology (JUST)
DAAD Research Grants for Doctoral Candidates and Young Academics and Scientists.

LIST OF PUBLICATIONS:

1. **Al-Salem, M**., & Serek, M. (2017). Expression analysis by RT-PCR of genes involved in ethylene synthesis and signal transduction in miniature roses. Scientia Horticulturae, 216, 22-28.

2. Atrooz, O. M., Omary, A. A., Khamas, W. A., Al-Shaikhly, A. A. Q. J., & **Al-Salem, M. M.** (2007). The effects of alcoholic extract of Arbutus andrachne on some biochemical parameters and visceral organs in rats. International journal of food science & technology, 42(9), 1046-1051.

3. **Al-Salem, M. M.,** & Karam, N. S. (2001). Auxin, wounding, and propagation medium affect rooting response of stem cuttings of Arbutus andrachne. HortScience, 36(5), 976-978.

4. Karam, N. S., & **Al-Salem, M. M.** (2001). Breaking dormancy in Arbutus andrachne L. seeds by stratification and gibberellic acid. Seed science and technology, 29(1), 51-56.