Efficacy of new inhibitors of ethylene perception in improvement of the display quality of miniature potted roses (*Rosa hybrida* L.)

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Dedicated to
my beloved brother

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Abbreviations

ACC	1-Aminocyclopropane-1-carboxylic acid
AdoMed	Adenosylmethionine
ACO	ACC oxidase
ACS	ACC synthase
AOA	Aminooxyacetic acid
AVG	Aminoethoxyvinylglycine
BA	Benzyladenine
CO_2	Carbondioxide
CP	Cyclopropene
3,3-DMCP	3,3-dimethylcyclopropene
DACP	Diazocyclopentadine
1-DCP	1-Decylcyclopropene
DNA	Deoxyribose nucleic acid
DNAase	Deoxyribonuclease
dNTP	Deoxyribonucleotide tri-phosphate
E. coli	*Escherichia coli*
1-ECP	1-Ethylcyclopropene
EDTA	Ethylene diamine tetra acetate
GC	Gas chromatograph
1-HCP	1-Hexylcyclopropene
LB medium	Luria-Bertani medium
1-MCP	1-Methylcyclopropene
3-MCP	3-Methylcyclopropene
NCBI	National center for biotechnology information
Nr	Never ripe (*LeETR3*)
1-OCP	1-Octylcyclopropene
RH	Relative humidity
RNA	Ribose nucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase polymerase chain reaction

SAM	S-adenosyl methionine
STS	Silver thiosulfate
TAE buffer	Tris-Acetate-EDTA buffer
Taq	*Thermus aquaticus*
Tris	tris-(hydroxymethyl) aminomethane

Genes/protein:

The symbol for genes is shown in italics and capitals (e.g. *ETR1*). The name of a mutation in a gene is italicized but not capitalized, e.g. *etr1-1*. The symbol for the protein encoded by the gene uses the same letters and numbers and is capitalized, but not italicized (e.g. ETR1)

ACO	ACC oxidase
ACS	ACC synthase
CTR	Constitutive triple response
EIL	EIN-like
EIN	Ethylene insensitive
ERF	Ethylene response factor
ERS	Ethylene response sensor
ETR	Ethylene resistant

Kurzfassung
Wirkung von neuen Inhibitoren der Ethlyenperzeption zur Verbesserung der Haltbarkeit von Topfrosen (*Rosa hybrida* L.)

Ethylen ist ein wichtiges Nacherndteproblem bei Topfrosen. So kommt es bei der Vermarktung durch das Verwelken der Blüten oder durch den Abwurf der Knospen, der Blüten und der Blätter zu Qualitätseinbußen. In dieser Arbeit wird die Wirkung und ein möglicher praktischer Einsatz von 1-MCP (1-Methylcyclopropen) Analogen, wie 1-OCP mit einer 8er-Carbon-Kette in der ersten Position und 1-DCP mit einer 10er-Carbon-Kette in der ersten Position für Topfrosen getestet. Die Sorte 'Lavender' Kordana mit einer hohen Ethylensitivität von Rosen Kordes, Sparrishoop, wurde, um die besten Bedingungen zu finden, im verkaufsfertigen Stadium mit unterschiedlichen Konzentrationen von 1-OCP, 1-DCP oder 1-MCP behandelt und anschließend, für das gesamte Experiment, kontinuierlich exogenem Ethylen ausgesetzt. Die Sorte 'Lavender' wurde dann mit der ethylen-tolerante Sorte 'Vanilla' verglichen. Der Versuch wurde vollkommen randomisiert mit 3 Wiederholungen und je 3 Töpfen pro Wiederholung durchgeführt. Ausgewertet wurde der Verlust der Blüten, Knospen und Blätter. Alle getesteten Konzentrationen der Chemikalien zeigten eine Wirkung im Vergleich zu den unbehandelten Kontrollpflanzen. 1-OCP und 1-DCP zeigte die beste Wirkung bei einer Konzentration von 1000 nl l^{-1}. Damit war eine fünfmal höhere Konzentration, als die zum Vergleich genutzte optimale Konzentration von 1-MCP (200 nl l^{-1}), notwendig. Die Wirksamkeit von 1-OCP und DCP wurde in Abhängigkeit zur Behandlungszeit und der Temperatur untersucht. Eine Behandlungszeit von 4 Stunden reichte sowohl für 1-OCP als auch 1-DCP aus, um eine optimale Haltbarkeit der Topfrosen zu erreichen. Eine längere Behandlungszeit führte zu keiner Verbesserung. Offenbar hatte auch die Behandlung der Topfrosen bei unterschiedlichen Temperaturen keinen Einfluss auf die Wirkung von 1-OCP und 1-DCP. So zeigten sich keine Unterschiede in einem Temperaturbereich zwischen 10°C und 20°C. Für die molekulargenetischen Untersuchungen wurden von der Sorte 'Vanilla' und 'Lavender' Blüten- und Blattproben nach der Behandlung mit 1-OCP, 1-DCP und 1-MCP nach 9 Tagen entnommen. Anschließend wurde die Expression von Genen der Ethylensynthese (5 unterschiedliche ACS Gene), der Ethylenperzeption (5 unterschiedliche Rezeptoren) und der Signaltransduktion (4 unterschiedliche Signaltransduktrionsgene) untersucht. Ein Signaltransduktionsgen (*RhERF*) wurde bei den Topfrosen erstmals isoliert. Das mögliche Polypeptid von *RhERF1* besitzt eine ERF Domain mit einer hohen Homologie zu bereits bekannten ERF Domains. Northernhybridisierungen wurden mit den Ergebnissen der RT-PCR verglichen. Jedoch zeigte sich, dass aufgrund der hohen Homologie zwischen den unterschiedlichen Ethylenrezeptoren eine Unterscheidung des Hybridisierungssignals schwierig war. Die RT-PCR Analysen zeigten, dass die Expression von allen untersuchten Genen, mit Ausnahme der Gene *RhACS1-2* und *RhETR4* in den Blütenblätter der Kontrollpflanzen 'Vanilla' und 'Lavender' durch Ethylen induziert wird. Dahingegen wurde nach einer Behandlung mit den Ethylenrezeptorinhibitoren (1-OCP und 1-MCP) eine Unterdrückung der Expression bei allen untersuchten Genen, mit und ohne Ethylen nachgewiesen. Jedoch konnte eine starke Expression der Gene *RhETR3*, *RhEIN3* und *RhEIL* nach einer Behandlung mit 1-DCP nachgewiesen werden. In der Abwesenheit von Ethylen zeigte sich eine starke Expression in den Blättern der 'Vanilla' Kontrollpflanzen bei allen Genen mit Ausnahme des Gens *RhACS1*. Nach der Behandlung mit den Ethylenrezeptorinhibitoren konnten keine Transkripte des untersuchten Gens in den Blättern von 'Vanilla' nachgewiesen werden. In den 'Vanilla' Blättern konnte eine Erhöhung der *RhEIN3* Transkripte nach der Behandlung mit den Ethylenrtezeptorinhiobitoren bei exogenem Ethylen nachgewiesen. Hingegen wurde bei den identischen Bedingungen eine Unterdrückung der *RhEIN3* Expression in den Blättern der Sorte 'Lavender' nachgewiesen werden. Möglicherweise sind die *RhEIN3* Transkripte ein limitierender Faktor bei der Ethylensignaltransduktion. Diese könnten dann während der Regulation der Blütenseneszenz auf transkriptionaler Ebene reguliert werden. In der Abwesenheit von Ethylen wird die Expression der Gene *RhACS1*, *RhETR3* und *RhERF1* unterdrückt. Eine ähnliche Wirkung zeigt auch 1-OCP, es inhibiert die Expression der Gene *RhACS1*, *RhCTR2* and *RhERF1*. 1-DCP inhibiert ebenfalls die Expression der Gene *RhEIN3* und *RhEIL*. Jedoch zeigt sich bei der Anwesenheit von Ethylen, nach einer Behandlung mit 1-MCP eine Expressionshemmung der Gene *RhACS1*, *RhETR4*, *RhCTR1-2*, *RhEIL*

und *RhERF1*. Eine ähnliche Wirkung zeigt 1-OCP. Diese Behandlung führt zu der Expressionsinhibierung der Gene *RhACS1*, *RhETR2*, *RhETR4*, *RhCTR1-2*, *RhEIN3* und *RhERF1*. Die Belandung mit 1-DCP unterdrückt die Expression der Gene *RhACS1* und *RhETR4*. Die Ergebnisse zeigen, dass die Expression der Gene der Ethylensynthese, der Ethylenperzeption und der Signaltransduktion nach einer Behandlung mit Ethylenrezeptorinihibitoren und bei Anwesenheit und nicht Anwesenheit von Ethylen, sowohl über eine positive als auch negative Feedbackreaktion bei den Sorten 'Vanilla' and Lavender', reguliert wird. Dies könnte sowohl durch die Pflanzenart als auch durch das untersuchte Gewebe beeinflusst werden. Zusammenfassend zeigen die Ergebnisse (physiologisch und molekulargenetisch), dass eine Behandlung mit Ethylenrezeptorinihibitoren die Haltbarkeit bei Topfrosen, durch die Verhinderung des Verlustes der Knospen, der Blüten und der Blätter, verlängert. Wobei jedoch 1-MCP eine bessere Wirkung, durch eine mögliche Unterdrückung der Gene der Ethylensynthese, der Ethylenperzeption und der Signaltransduktion zeigt, als 1-OCP und 1-DCP.

Schlüsselwörter: ACC Synthase, Ethylenperzeption und Signaltransduktion, Ethylen, Ethyleninhibitoren, Haltbarkeit, Genexpression, *Rosa hybrida*, Seneszenz

Abstract
Efficacy of new inhibitors of ethylene perception in improvement of display quality of miniature potted roses (*Rosa hybrida* L.)

Ethylene is an important postharvest problem in miniature potted roses that leads to loss of quality during marketing by accelerating flower senescence, and bud, flower and leaf drop. The effect of pretreatments with analogues of 1-MCP, 1-OCP substituted with an 8-carbon chain in the 1-position and 1-DCP with a 10-carbon chain, and 1-MCP were studied to possibly come up with a potential commercial approach for improving display quality of miniature potted roses. 'Lavender' Kordana breeding line from Rosen Kordes, Sparrishoop, which is sensitive to ethylene, were grown and pretreated after harvest with different levels of 1-OCP, 1-DCP, 1-MCP or air and continuously exposed to exogenous ethylene throughout the experimental period to find the best conditions for 'Lavender' as compared with 'Vanilla', which is insensitive to ethylene. They were then arranged in a completely randomized design comprising 3 replications per treatment of 3 pots per replication and evaluated for bud and flower drop as well as leaf drop. All tested levels of all chemicals were effective when compared to untreated (control) plants. 1-OCP and 1-DCP were the most effective at concentrations 1000 nl l^{-1}, which was five times higher than the concentration of 1-methylcyclopropene (1-MCP) (200 nl l^{-1}) used as a standard. Exposure time of 4 h for both 1-OCP and 1-DCP was sufficient to improve display life of miniature roses and longer exposures did not have any additional beneficial effect. Apparently, exposing miniature potted roses to various temperatures did not have an influence on the performance of both 1-OCP and 1-DCP, which were effective at temperatures of 10°C to 20°C. Samples of 'Vanilla' and 'Lavender' petals and leaves from the best condition of pretreatment of 1-OCP, 1-DCP and 1-MCP or air were taken for molecular studies in investigating the expression of genes responsible for ethylene biosynthesis enzyme (5 different ACS genes), ethylene perception (5 different receptors) and signal transduction pathway (4 different transcription regulating genes) after 9 days of continuous exposure to exogenous ethylene. One transcription regulating gene (*RhERF1*) was isolated from miniature rose. The putative polypeptide of *RhERF1* had an ERF domain, which shares high homology with other reported *ERF* domains. Northern blot hybridization analysis was used to compare with results of RT-PCR. However, it was difficult to distinguish the hybridization signal of ethylene receptors between *RhETR1* and *RhETR2*, which were high homolog. RT-PCR analysis revealed that ethylene induced the expression of all genes investigated in control 'Vanilla' and 'Lavender' petals except *RhACS1-2* and *RhETR4*, respectively, while pretreatments of ethylene receptor inhibitors (1-OCP and 1-MCP) suppressed the expression of all genes in the presence or absence of ethylene. However, strong expression of *RhETR3*, *RhEIN3* and *RhEIL* transcripts was detectable in 'Lavender' petals pretreated with 1-DCP. In the absence of ethylene, there was strong expression of all genes in control 'Vanilla' leaves but no expression of *RhACS1*, while the accumulation of all genes was eliminated after pretreatments of 'Vanilla' leaves with ethylene receptor inhibitors. However, the level of *RhEIN3* mRNA was upregulated by pretreatment of ethylene receptor inhibitors in 'Vanilla' leaves in the presence of ethylene while the accumulation of *RhEIN3* mRNA was suppressed in 'Lavender' leaves. *RhEIN3* transcript is probably rate-limiting for ethylene perception and signal transduction pathway, which is regulated in leaves during flower senescence at the transcriptional level. In the absence of ethylene, 1-MCP suppressed the expression of *RhACS1*, *RhETR3* and *RhERF1* transcripts. Similarly, 1-OCP inhibited the expression of *RhACS1*, *RhCTR2* and *RhERF1* transcripts. Likewise, 1-DCP inhibited the expression of *RhEIN3*, *RhEIL* genes. However, in the presence of ethylene, pretreatment of 'Lavender' leaves with 1-MCP suppressed the expression of *RhACS1*, *RhETR4*, *RhCTR1-2*, *RhEIL* and *RhERF1* transcripts. Similarly, 1-OCP treatment inhibited the expression of *RhACS1*, *RhETR2*, *RhETR4*, *RhCTR1-2*, *RhEIN3* and *RhERF1* transcripts. Pretreating 'Lavender' leaves with 1-DCP suppressed the expression of *RhACS1* and *RhETR4* transcripts. These results indicated that the expression of genes for ethylene biosynthesis enzyme, ethylene perception and signal transduction pathway was regulated by both positive and negative feedback regulation mechanism in 'Vanilla' and Lavender' pretreated with ethylene receptor inhibitors in the presence or absence of ethylene. This might depend on plant species and tissues under investigation. Physiologically and molecularly, these results suggest that pretreatments with

inhibitor of ethylene receptor improved the display life of miniature roses by delaying bud, flower and leaf drop, whereas 1-MCP is more effective than 1-OCP and 1-DCP, possibly by suppressing the expression of some genes for ethylene biosynthesis enzyme, ethylene perception and signal transduction pathway.

1 General Introduction

1.1 Ethylene and flower senescence

Senescence can widely be defined as the combination of events that lead to the death of cells, tissues or organs (Reid and Wu, 1992). This process occurs at many stages during the development of an organism and at many levels (Noodén et al., 1997). It is mediated by a series of highly coordinated physiological and biochemical changes, such as increased activity of hydrolytic enzymes, degradation of macromolecules, loss of cellular compartmentation and increase in respiratory activity. These changes are related to changes in gene expression and synthesis of protein (Borochov and Woodson, 1989; Van Altvorst and Bovy, 1995).

Flower senescence is a common cause of quality loss and reduced vase life of flowering plants and cut flowers (Serek and Reid, 2000a). Senescence in many flowers is accompanied by pollination promoting the production of ethylene which ultimately causes petal wilting (Nichol, 1977), abscission and sleepiness (florets failed to re-open) of petals (Marousky and Harbaugh, 1979) and a climacteric increase in ethylene production (Nichols, 1968; Singh and Moore, 1994). This is induced by several factors, e.g., water stress (Sankat and Mujaffar, 1994), carbohydrate depletion (Ketsa, 1989), microorganisms (Witte and Van Doorn, 1991), and ethylene effects (Wu et al., 1991a, b). The phytohormone ethylene plays a vital role in regulation of flower senescence in many species. Additionally, ethylene-induced senescence in some sensitive species may result from the production of endogenous ethylene or from exposure to exogenous ethylene (Van Altvorst and Bovy, 1995; Halevy and Mayak, 1979). The action of ethylene is based on two types of responses: the response to a change in the concentration of ethylene, and the response to a change in the sensitivity of tissue to ethylene (Sato-Nara et al., 1999). Certain plants are ethylene-sensitive and flowers of these plants deteriorate rapidly in the presence of ethylene. Ethylene can be produced by almost all parts of plants, although the production relies on the type of tissue and stage of development (Müller and Stummann, 2003). In carnation, a large amount of ethylene is synthesized several days after full opening of the flower during natural senescence (Manning, 1985; Peiser, 1986; Woodson et al., 1992), or several hours after compatible pollination (Nichols, 1977; Nichols et al., 1983; Larsen et al., 1995) or treatment with exogenous ethylene (Borochov and Woodson, 1989; Wang and Woodson, 1989). The increased

ethylene production accelerates in-roll of petals resulting in wilting of the flower. Flowers develop the early stages of wilting symptoms when treated with ethylene and later disappear when ethylene treatment is stopped. This suggests that ethylene is required beyond the appearance of early visual symptoms to uphold the "senescence syndrome" (Mayak and Kofranek, 1976).

1.2 Exogenous ethylene

Senescence of climacteric flower petals is associated with an increased rate of ethylene production and respiration, concomitant with the onset of petal wilting. Exposure to exogenous ethylene promotes senescence of climacteric flower petals with activation of ACC synthase and/or ACC oxidase (Borochov and Woodson, 1989).

Woltering and Doorn (1988) studied petal senescence in mature flowers of 93 species from 22 families. Most of the flower families (Geraniaceae, Libiatae, Ranunculaceae, Rosaceae and Scrophulariaceae) showed initial abscission in response to ethylene except for a few families (Caryophyllaceae, Campanulaceae, Malvaceae and Orchidaceae), which showed wilting as their primary senescence symptom.

Moreover, Woltering and Doorn (1988) observed that abscission in Rosaceae is the initial symptom of flower senescence. Further evidence supporting this view has found that bud abscission of 'Victory Parade' roses occurs when plants are sensitive to ethylene (Serek, 1993). However, in miniature potted roses, petal wilting as a form of flower senescence is more obvious than petal abscission (Müller *et al*. 1998). The aging process of the flowers is apparently accelerated by exogenous ethylene. Increasing amounts of exogenous ethylene in postharvest environments also induces a variety of symptoms (petal abscission, leaf and bud drop) in rose cultivars, and there are marked differences in sensitivity and response to ethylene among cultivars (Müller *et al*., 1998).

1.3 Endogenous ethylene

Ethylene clearly influences natural flower senescence as an integral part of the aging process in *Ipomoea* and *Hibicus* and serves to accelerate aging but may not be the initial causative agent. This accelerating effect of ethylene is apparently related to the autocatalytic nature of the ethylene biosynthetic system where ethylene stimulates its own synthesis in both flowers and ripening of climacteric fruits (Kende and Baumgartner, 1974; Kende and Hanson, 1976; Beyer and Sundin, 1978; Woodson *et al.*, 1985; Halevy and Mayak, 1979). In carnation flowers, an increase in ethylene production rate, similar to the climacteric associated with fruit ripening, coincides with the first visible signs of senescence by petal in-rolling (Peiser, 1986; Mayak and Tirosh, 1993). During the aging of petals, the transition to autocatalytic ethylene production results from a change in tissue responsiveness to ethylene and an increase in their capacity to respond to exogenous ethylene by the induction of autocatalytic ethylene production, indicating a gradual release of the ethylene biosynthetic pathway from restriction (Wang and Woodson, 1989). Several studies have been shown that rose flower senescence is regulated by ethylene. Many cultivars of miniature roses also show a climacteric rise in ethylene production during flower senescence, even though large differences are found among cultivars. In some cultivars, flower senescence is accompanied by a clear climacteric rise in ethylene production, and by a moderate or low ethylene production in others. Longevity of miniature roses in the absence of exogenous ethylene is presumably a function of endogenous ethylene production. For example, the short life of individual flowers in 'Bronze' is associated with a clear climacteric peak in ethylene production, similar to that found in typical climacteric flowers. The excellent longevity of 'Charming Parade' in an ethylene-free environment, however, is related to a very low ethylene production of flowers (Müller and Stummann, 2003).

1.4 Pollination-induced senescence

Pollination in many flowers causes ethylene biosynthesis and developmental changes such as ovary growth and petal wilting and abscission. These processes are induced by a translocated signal that precedes the growing pollen tube signal, a compatible pollination, to ovary and petals. In addition, styles are sources of high ethylene production, which can be activated by pollination (Jones and Woodson, 1999a; Nichols, 1977). Two or three phases of pollination-induced ethylene

production occur in petunia, tobacco, and carnation. The first peak of ethylene is detectable within a few minutes of pollination, then sustains climacteric of ethylene production and follows in response to a compatible pollination and hastens senescence of the style and petals. An unidentified senescence signal appears to be translocated to other flower parts, which become active sites of autocatalytic ethylene production within a few hours of pollination (Holden *et al.*, 2003). Application of amino-oxyacetic acid (AOA), an inhibitor in ethylene biosynthesis, blocks pollination-induced ethylene production in orchid flowers, decreases ovary growth and delays senescence (Ketsa and Rugkong, 2000).

1.5 Non-ethylene-mediated senescence

Senescence of the petals of many cut flowers such as Compositae, Iridaceae, Liliaceae and also most of the important geophytes appears not to be related to ethylene (Reid, 1989; Woltering and Van Doorn, 1988).

Flower senescence in *Hemerocallis*, daylily, is ethylene-independent. The senescence of these flowers is not accelerated by exposure to exogenous ethylene, nor delayed by inhibitors of ethylene biosynthesis or by ethylene antagonists (Lukaszewski and Reid, 1989). The major events that occurred in the ethylene-unresponsive daylily are an early decline in phospholipid synthesis, an increase in cell permeability that leads to an increase efflux of sugars and ions, a respiration climacteric, early wilting and then autolysis of petal tissue. Moreover, flower senescence in daylilies is accompanied by both the decline in protein content and the breakdown of specific proteins in the petals. The inhibition of flower senescence by cyclohexamide (CHI) could maintain protein content and protein population of the petals. These effects of CHI may require de novo protein synthesis in these species during senescence and are key factors controlling petal senescence in a species where the regulation of this event appears to be independent of ethylene (Bieleski and Reid, 1992; Lay-Yee *et al.*, 1992).

Gladiolus, like other geophytes in Liliaceace, is an ethylene-insensitive flower (Lay-Yee *et al.*, 1992). Ethylene is not a factor in floret senescence since wilting of florets is not accelerated by exposure to ethylene, nor delayed by ethylene antagonists (Serek *et al.*, 1994a).

In *Cyclamen persicum* Mill flowers, senescence is not associated with an increase in ethylene production. Flowers do not respond to exogenous ethylene even when exposed to very high concentrations of the gas. Pollination, however, induces a dramatic increase in ethylene evolution. This presumably indicates that the promotion of senescence (corolla drop) in pollinated cyclamen flowers is mediated by ethylene (Halevy *et al.*, 1984).

1.6 Ethylene biosynthesis

The pathway of ethylene biosynthesis in plants has been explained (Yang, 1985; Van Alvorst and Bovy, 1995).
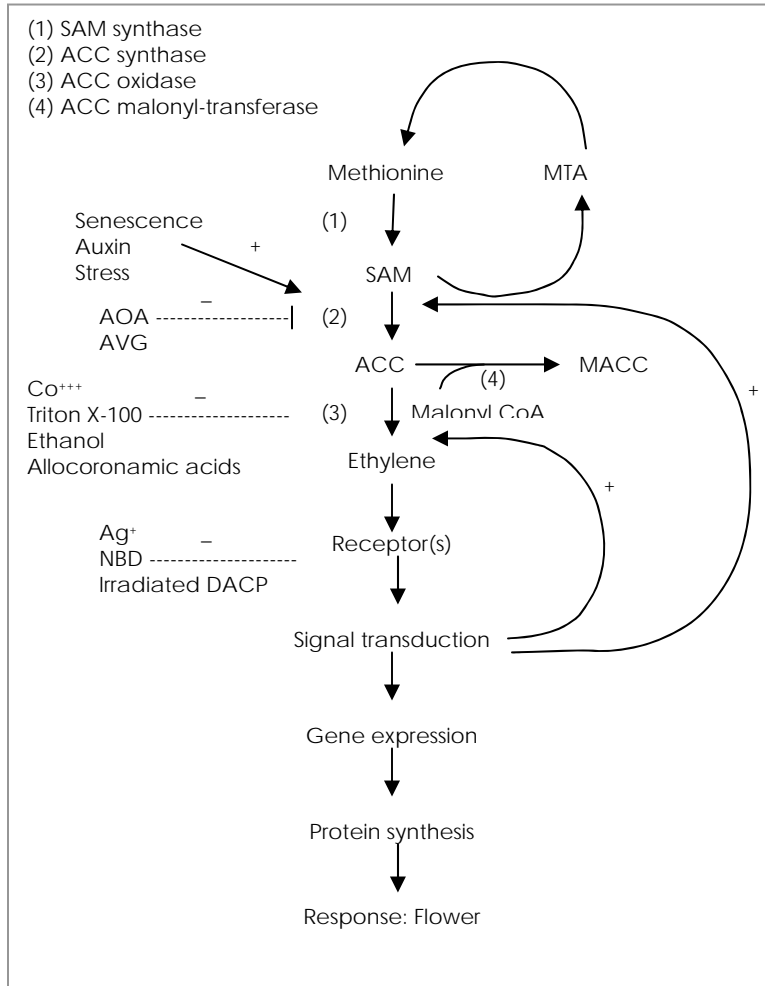
Fig. 1 Ethylene biosynthesis pathway by Van Alvorst and Bovy (1995).

In higher plants, ethylene is synthesized from methionine via a pathway involving the conversion of S-adenosylmethionine (SAM) to 1-amino cyclopropane-1-caboxylic acid (ACC) and the oxidation of ACC to ethylene. The enzyme ACC synthase converts SAM to ACC while ACC oxidase catalyzes the conversion of ACC to ethylene, HCN and CO_2. In addition, methionine is regenerated in the Yang cycle (Adam and Yang, 1979) (Fig. 1).

1.6.1 ACC

The intermediate precursor of ethylene in higher plants is 1-aminocyclopropene-1-carboxylic acid (ACC) (Adam and Yang, 1979). The endogenous ACC level in various flower parts increases during senescence (Veen and Kwakkenbos, 1982; Nichols *et al*., 1983; Hsieh and Sacalis, 1986). Application of ACC stimulates wilting in whole carnation flowers (Veen and Kwakkenbos, 1982). Exposure to ethylene of isolated carnation petals, separated into upper and basal parts, shows that the majority of ethylene production is evolved from the basal part of the petals. Endogenous ACC content in the basal portions of senescing carnation petal is 3 to 5 times higher than in the upper parts. The upper portions respond to ethylene by delaying wilting and much lower ethylene production. Application of ACC to the upper portion of senescing petals increases their ethylene production (Mor *et al*., 1985). During flower senescence, ACC is translocated from the basal part where it is synthesized to the upper part where it is converted into ethylene (Overbeek and Woltering, 1990). In addition, the endogenous ACC content of pollen correlates with the amount of ethylene that is produced by petunia styles immediately after pollination (Singh *et al*., 1992). The detection of ACC in petunia pollen indicates that ACC diffusing from pollen might be responsible for early ethylene production, initiating autocatalytic ethylene production (Stead, 1985).

1.6.2 ACC synthase and ACC oxidase

The biosynthesis of ethylene in flower tissues is under strict metabolic regulation and is subject to induction by a variety of signals including emasculation, pollination, wounding, auxin, abscissic acid and environment stress (Borochov and Woodson, 1989). The conversion of SAM to ACC is catalyzed by the peridoxal phosphate-requiring enzyme ACC synthase, which represents the rate-limiting step in ethylene biosynthesis in many plant tissues (Kende, 1989, 1993). All evidence

indicates that ACC synthase is a cytoplasmic enzyme (Kende, 1993). Boller *et al.* (1979) found that ACC synthase is a soluble enzyme in tomato fruits. The final step in the ethylene biosynthetic pathway is catalyzed by ACC oxidase, formerly referred to as the ethylene-forming enzyme (EFE) (Kende, 1989, 1993). ACC oxidase is located in the cell walls of apple and tomato fruit cells. In apple, the enzyme is actually located at the external face of the plasma membrane (Ramassamy *et al.*, 1998; Rombaldi *et al.*, 1994). ACC oxidase requires ascorbate, Fe^{2+}, and CO_2 which act as essential cofactors (Smith and John, 1993a, b). Both ACS and ACO enzymes from a number of different species are encoded by multigene families whose members are differentially regulated in a tissue-specific manner by a variety of developmental cues, mechanical and environmental stresses, and by ethylene, which stimulates its own biosynthesis (Kende, 1993; Fluhr and Mattoo, 1996).

1.7 Strategies to breed ethylene-insensitive flowers

The development of ethylene-resistant cultivars that are mutant with impaired ethylene biosynthesis or sensitivity has been studied in carnations as a model system leading to a more focused breeding effort in the genetic improvement of postharvest longevity of these flowers. The extended vaselife genotypes are responsive to genetic differences by synthesis and response to ethylene. For example, genotype 799 is impaired in its ability to respond to ethylene by increasing ethylene production or premature petal senescence. The second genotype, 87-37G-2, appears to represent a mutation affecting the synthesis of ethylene but not ethylene responsiveness. The third genotype, 81-2, is able to synthesize ethylene in response to ethylene treatment but failed to produce ethylene during normal aging. This pattern shows that these flowers fail to produce elevated ethylene during aging but respond to exogenous ethylene by increased ethylene synthesis and premature senescence (Brant and Woodson, 1992). Similar to Sandrosa carnations, these flowers do not exhibit an autocatalytic increase in ethylene production nor do they develop petal in-rolling, but the sensitivity to ethylene diminishes with age, whereas other flowers show increased sensitivity to ethylene with age (Mayak and Tirosh, 1993). In 'killer' carnations that show non-climacteric behavior, the lack of ethylene production is due to a limited-available ACC, likely resulting from low ACC synthase activity and failure to convert ACC into ethylene because of restricted ACC oxidase activity (Serrano *et al.*, 1991).

'Sandra' and 'Chinera' carnation flowers last about twice as long as those of 'White Sim' whose senescence closely correlates with the normal climacteric of respiration and ethylene production and is accompanied by a marked increase in ACC content and ACC oxidase activity. In contrast, the long vase life of Sandra flowers is due to inhibition, under normal conditions, of the pathway for ethylene biosynthesis (Wu *et al*., 1989, 1991a). Although behavior of non-climacteric carnation 'Sandra' fails to produce elevated ethylene during aging, it responds to exogenous ethylene by increasing ethylene synthesis and premature senescence. 'Chinera' flowers not only produce less ethylene during natural senescence, but are also much less sensitive to ethylene (Wu *et al*., 1991b).

1.8 Ethylene sensitivity

Various processes during plant development are determined both by ethylene production and by the tissue responsiveness to ethylene. Therefore, differences in sensitivity to hormones are the main factor controlling plant response (Trewavas, 1982). During senescence, changes in a climacteric rise in ethylene production and a gradual increase in ethylene sensitivity occur in the corollas of climacteric flowers such as carnation and petunia (Nichols, 1968; Halevy and Mayak, 1981; Whitehead and Halevy, 1989). In addition, pollination of these flowers leads to an acceleration of senescence involved in a marked stimulation of ethylene synthesis and a sudden increase in sensitivity of the corolla to ethylene (Whitehead and Halevy, 1989; Whitehead and Vasijevic, 1993; Porat *et al*., 1994; Halevy *et al*., 1996).

The increase in ethylene sensitivity following pollination is independent of endogenous ethylene production since it occurs in flowers treated with AOA, which prevents the increase in ethylene synthesis. An increase in ethylene sensitivity following pollination is thought to render the tissue to respond to low basal ethylene, thus inducing the later autocatalytic increase in ethylene production. This is also related to its ability to increase ethylene binding by altering certain membrane properties which could lead to an increase in the availability of ethylene binding sites (Porat *et al*., 1993, 1994, 1995b; Whitehead and Vasijevic, 1993). It is clear that ethylene cannot be the pollination signal inducing sensitivity to ethylene (Porat *et al*., 1993, 1994, 1995b).

The possibility that the sensitivity factor is short-chain saturated fatty acids (C_7-C_{10}) has been postulated (Whitehead and Halevy, 1989). Application of these acids to the stigmas of petunia

and carnation flower results in a sudden increase in ethylene sensitivity and a marked acceleration of senescence (Whitehead and Vasijevic, 1993). Treatment of Dutch iris (*Iris*) 'Sapphire Beauty' bulb with the short-chain saturated fatty acid octanoic acid (C_8) increases ethylene sensitivity by stimulating flowering (Botha *et al*., 1998).

However, in carnation, petunia and cymbidium (*Cymbidium*), short-chain saturated fatty acid does not play an important role in flower senescence and ethylene sensitivity (Wotering *et al*., 1993).

1.9 Statement of the problem

Miniature potted roses are popular greenhouse crops in many parts of the world. In the 1980s, there was an introduction of new varieties that have had a dramatic impact on the European and North American markets. Current annual world production is estimated at 60-80 million pots in Europe and 26 million pots in the United States. Major centers of production include Denmark, The Netherlands, the United States, Canada and Japan with production also in France, Germany and Italy (Pemberton *et al*., 2003; USDA, 2003: http://www.ars.usda.gov). The acceptability and trade value of miniature potted roses can be influenced by postharvest longevity, where in Denmark, extensive efforts have been taken to improve the quality and increase the production of these plants, for example with a breeding program (Serek and Andersen, 1993, Müller *et al*., 1998). Loss of quality during postharvest and marketing in miniature potted roses are important problems caused by ethylene action (Serek, 1993; Serek *et al*., 1994c), such as accelerated flower wilting, leaf yellowing, bud and leaf abscission and infection by grey mould (*Botrytis cinerea*). It is assumed that ethylene is not the primary cause of leaf yellowing in miniature potted roses, but that it accelerates flower senescence as well as bud, flower and leaf drop (Müller and Stummann, 2003). Variation in postharvest life of miniature roses is partly the result of differences in endogenous ethylene during flower senescence, stress-induced ethylene production and sensitivity to exogenous ethylene. Ethylene sensitivity has important implications during the transport and handling of potted roses in supermarkets and other areas where the air is commonly contaminated with ethylene (Müller *et al*., 1998, 2000a). In ethylene-contaminated air, rose cultivars with high sensitivity to exogenous ethylene exhibit a short flower life (Müller *et al*., 2000a). Ethylene action involves binding to a specific receptor (Schaller and Breecker, 1995; Sisler and Serek, 1997). Since ethylene inhibitors can block the plant tissues from endogenous

and exogenous ethylene, they are considered very potent for horticultural use (Sisler and Serek, 1997; Feng *et al*., 2000). 1-MCP and some new putative inhibitors of ethylene action, which are structurally analogous to 1-MCP containing a longer side chain at 1-position (1-OCP and 1-DCP), have been found to be effective in protecting cut flowers against ethylene (Kebene *et al*., 2003a, b) and delaying the sensitivity of bananas to ethylene (Sisler *et al*., 2003). In addition, the expression patterns of the genes for the ethylene biosynthetic enzyme ACC synthase, and for ethylene receptors and signal transduction pathway during flower development and senescence processes seem essential to understanding ethylene response. Understanding the roles of the various ethylene-relevant genes in flower development may lead to an ability to selectively block processes in flower senescence that are economically detrimental, and to improve display life using both conventional breeding and biotechnological engineering.

1.10 Objectives

1. To investigate the effect of extended chain length of the activity of inhibitors of ethylene receptors 1-OCP and 1-DCP as compared with 1-MCP, in preventing the effects of ethylene on display quality of miniature potted roses.
2. To determine the optimum concentrations, exposure time and temperature at which 1-OCP and 1-DCP effectively improve the longevity of miniature potted roses.
3. To determine the effectiveness of 1-OCP, 1-DCP and 1-MCP between miniature potted rose cultivar 'Lavender' with sensitivity to ethylene and 'Vanilla' with long-lasting vase life.
4. To investigate the effect of 1-OCP, 1-DCP and 1-MCP on the expression of the genes for the ethylene biosynthetic enzymes, ethylene perception and signal transduction pathway in miniature potted roses.

2. Efficacy of new inhibitors of ethylene perception in improvement of display quality of miniature potted roses (*Rosa hybrida* **L.**)

Abstract

1-Octylcyclopropene (1-OCP) and 1-Decylcyclopropene (1-DCP), which act as ethylene receptor inhibitors and are analogues to 1-MCP, but are substituted with longer carbon chains in the 1-position, were investigated in miniature potted rose cultivar 'Lavender'. All tested levels of both chemicals were protected as compared to untreated plants. 1-OCP and 1-DCP were the most effective at concentrations of 1000 and 1500 nl l^{-1}, which was five times higher than the concentration of 1-methylcyclopropene (1-MCP) (200 nl l^{-1}) used as a standard. The effectiveness of 1-OCP and 1-DCP was a function of time and temperature. At short (2 h) exposure times, the plants were highly sensitive to ethylene. Exposure time of 4 h for both 1-OCP and 1-DCP was sufficient to improve display life of miniature roses and longer exposures did not have any additional beneficial effect. Apparently, exposing miniature potted roses to various temperatures did not have an influence on the performance of either 1-OCP or 1-DCP. They were effective at temperatures between 5 to 20°C. The effectiveness of this group of compounds on the display quality of miniature roses is discussed.

Key words: 1-DCP, Ethylene receptor inhibitors, 1-MCP, 1-OCP, Postharvest performance

2.1 Introduction

Ethylene has been shown to play a central role in physiological and developmental processes such as seed germination, growth, flower initiation, organ abscission, fruit ripening, senescence of leaf and flowers and response to pathogen attack (Abeles *et al.*, 1992). Ethylene binding would be mediated by a transition metal cofactor (Burg and Burg, 1967). The isotopic competition technique can explain the presence of a specific ethylene binding site in carnation petals exposed to ^{14}C-C_2H_4. A substantial proportion of the bound radioactive ethylene could be displaced by the addition of unlabelled ethylene, identifying a specific binding site. Treatment with ethylene action inhibitors silver or 2,5-NBD to flowers reduces binding activity (Sisler *et al.*, 1983). In addition, the binding of ethylene in petal tissues is significantly higher for young carnation petals than for older flowers. The peak in ethylene binding precedes the climacteric-like rise in ethylene production. Ethylene binding changes in senescent flowers because the number of binding sites and the affinity for ethylene in the older tissue decreases (Brown, 1986).

Copper is the metal required for the high affinity ethylene binding that receptors display (Rodriguez *et al*, 1999). Therefore, the binding of ethylene to its membrane associated with receptor sites is a crucial step in its action (Goren *et al.*, 1984). Additionally, the number of receptors could have an essential role in determining the sensitivity to ethylene and the type of response that is elicited. When ethylene binds to the receptors, it induces a series of events that initiate a response to the hormone (Tian *et al.*, 1997).

2.1.1 Compounds interacting with the ethylene receptor in plants

2.1.1.1 Ethylene and ethylene analogues

Compounds such as ethylene, some olefins, propylene, acetylene, carbon monoxide and isocyanides, which bind to the receptor, are capable of triggering ethylene-induced changes in the growth and development of plants. Ethylene and its analogues are thought to interact with a metal-containing receptor (Sisler and Wood, 1988; Sisler *et al.*, 1990; Sisler and Serek, 2003). Of the group, ethylene is considerably more effective than the others, which are not used for some experimental research, but give some insight into how ethylene behaves (Sisler and Serek, 2001).

2.1.1.2 Competitive ethylene antagonists

Compounds preventing an ethylene response interact with the receptor and compete with ethylene for binding. A single exposure of plant tissue to these compounds is enough to prevent binding of ethylene because they remain bound for a long period of time, with even high levels of ethylene not inducing any action (Sisler and Serek, 2003).

2.1.2 Mode of action of compounds blocking the receptor

Competitive ethylene antagonist compounds presumably bind to a metal in the ethylene receptor. They compete with ethylene for the receptor and prevent the ethylene receptor from binding in treated tissues. While they are bound, ethylene cannot bind (Sisler and Serek, 1997).

Both those compounds inducing a response and those blocking the receptor should bind to the supposed metal (M) on the receptor and withdraw electrons from the metal, which causes a rearrangement or change in ligand (L_1-L_5) on the metal. Then, ethylene (E) is likely to come off of the metal in a few minutes while other inhibitory compounds that block receptors appear to take at least several hours or, in some, many days to leave (Sisler and Serek, 1999) (Fig. 2). Ethylene leaves the complex and causes it to become active probably by ligand arrangement. Ethylene then would not be a part of the active complex, but the initiator of its formation. While the inhibitory compounds act in a similar manner to ethylene, they do not move away from the complex, thus an active complex is not formed (Sisler and Serek, 1997), which accounts for the mode of action of ethylene and 1-MCP that blocks ethylene responses (Sisler and Serek, 1997).

Proposed model:

Fig. 2. Proposed model for action of ethylene and 1-methylcyclopropene (1-MCP) on the ethylene receptor (Sisler and Serek, 1997).

It is believed that the ligands could be in a protein group such as histidine, methionine, thiol groups, tyrosine groups and in a hydrophobic environment such as a membrane. Perhaps the double bonds in unsaturated fatty acids, on a single protein forming a cross link between two proteins, could be all ligands (Sisler and Serek, 1999).

2.1.3 Ethylene antagonists inactivating the receptor for an extended period of time

Silver ion, applied as Silver thiosulfate (STS), is a potent anti-ethylene compound in various plants. Silver acts through the irreversible interaction with ethylene binding sites (Sisler *et al.*, 1986; Veen, 1986; Rodriguez *et al*, 1999). STS is in widespread commercial used to inhibit effects of ethylene and prolong vase life in many ornamentals including orchid (*Cattleya*) 'Loiuse Georgeianna' (Beyer, 1976), carnation (Veen, 1979), sweet pea (*Lathyrus odoratus*) (Mor *et al.*, 1984), potted Christmas cactus (*Schlumbergera truncate*) (Serek and Reid, 1993) and potted rose (*Rosa hybrida*) (Serek, 1993). However, as Silver is a heavy metal, it cannot be used on food and feed, and many countries prohibit its use (Veen, 1986).

Cyclic olefins appear to block ethylene responses rather than to induce a response. All of these compounds are released from the receptor and diffuse from the binding site over a period of

several hours. 2,5-norbonadiene (2,5-NBD) is found to compete with ethylene for binding sites in carnation petals, but it requires continuous exposure to be effective (Wang and Woodson, 1989; Sisler and Serek, 1999). Another compound in this group is *trans*-cyclooctene, which is much more effective, in terms of concentration, than 2,5-NBD. However, the practical use of this compound is limited by a very pungent and objectionable odor (Sisler *et al.*, 1990).

Diazocyclopentadiene (DACP) is a putative photoaffinity label for ethylene binding sites. These compounds form highly reactive carbons under UV light, called diazo-compounds. Under UV light, nitrogen gas is split from the molecule leaving a carbine, which almost immediately reacts with many other compounds (Sisler and Serek, 2003). It binds irreversibly to the ethylene receptor or at least remains bound for many days to reduce tissue responses to ethylene in mung bean sprouts and tobacco leaves (Sisler and Blankenship, 1993), potted roses (*Rosa hybrida*) (Serek *et al.*, 1994b) and tomato fruit (Tian *et al.*, 1997). A major problem with DACP is that it is explosive in high concentrations, which limits its commercial usefulness (Sisler and Serek, 1997).

A series of cyclopropenes, cyclopropene (CP), 1-methylcyclopropene (1-MCP), 3-methylcyclopropene (3-MCP) and 3,3-dimethylcyclopropene (3,3-DMCP), have been prepared and tested by using banana fruit as an assay system. Based on the substitution of the cyclopropene ring in positions 1, 2 and 3, all compounds are effective in preventing banana from exogenous ethylene but none are more effective, concentration-wise, than 1-MCP (Sisler *et al*, 1996a, b, 1999, 2001). CP and 1-MCP act at low concentrations. In banana, they are effective around 0.5 nl l^{-1}. The required concentration of 3,3-DMCP is about 1000 times as high as CP and 1-MCP. It is effective in the μl l^{-1} range and also protects banana for only 7 days (Sisler *et al.*, 1996a, b). 3-MCP is also effective at higher concentrations than for 1-MCP. In banana, about 2.5 times as much 3-MCP is needed (Sisler *et al.*, 1999).

In the substitution on the cyclopropene ring, both the concentration required for inactivation and duration of binding appear to be influenced by steric and inductive effects (Sisler *et al.*, 1996b, 1999, 2001). The presence of the methyl groups in these compounds is associated with steric effect. Flat molecules (cyclopropene, 1-MCP and 3-MCP) are more active than compounds with the methyl groups isolated from the double bond. Inductive effect, however, is caused by methyl.

A methyl group releases electrons into the cyclopropene ring and is capable of relieving the strain related to anti-ethylene effects of compounds in plants (Sisler *et al.*, 2001).

The position of the methyl group is more important. In 1-MCP the methyl group is adjacent to the double bond, thus allowing an allylic-type of arrangement. This may interact with the ethylene receptor more rapidly than isolating a double bond in 3-MCP and result in a low concentration requirement for a given time exposure (Sisler, 1999).

Stability is an important factor in these compounds in considering commercial use. CP is unstable in the liquid phase even at -78°C, and in a dilute gas phase seems to polymerize at room temperature. 1-MCP, 3-MCP and 3,3-DMCP are relatively stable (Sisler *et al.*, 1996a, b; Sisler *et al.*, 2001).

1-MCP, one of the most useful compounds among substituted CPs, is a non-toxic compound, stable at room temperature, active at relatively low concentration that provides protection for a longer period of time up to 12 days after a single exposure, without any detectable odor. Moreover, in the floriculture industry 1-MCP can be used as a replacement of silver thiosulfate (STS), which is considered to be toxic (Sisler and Serek, 2001, 2003). Other CPs substituted with the methyl group in the 1-position have been developed and tested as ethylene antagonists (Sisler *et al.*, 2001).

1-MCP prevents damage from exogenous ethylene in many potted plant species including heimalis begonia 'Najada' and 'Rosa' and tuberous begonia (*Begonia x tuberhybrida*), rose 'Victory Parade' (*Rosa hybrida*) (Serek *et al.*, 1994c), kalanchoe 'Tropicana' (*Kalanchoe blossfeldiana*) (Serek and Reid, 2000b), Christmas cactus (*Schlumbergera truncate*) and bellflower (*Campanula carpatica*) (Serek and Sisler, 2001) and in some countries has already been approved for use in edible crops (Blankership and Dole, 2003). However, the effect of 1-MCP on ethylene-induced petal abscission in ivy geranium 'Pink Blizzard' (*Pelargonium peltalum*) is transient (Cameron and Reid, 2001).

Recently, a series of cyclopropenes substituted with a methyl group in the 1-position, analogues of 1-MCP, protected for longer periods of time than those substituted in other positions (Sisler *et al.*, 2003; Sisler and Serek, 2003). When the chain length is extended to more than four carbons,

the minimum concentration requirement declines (Sisler *et al.*, 2001), however, 2 or 3 carbon side chains substituted at the 1-position, 1-ethylcyclopropene (1-ECP) and 1-propylcyclopropene (1-PCP), are still effective blockers of ethylene receptors and are able to inhibit ethylene action in a wide scope of systems. These include climacteric fruits like avocado and tomato, 'the triple response' in etiolated peas and abscission of citrus leaf explants (Feng *et al.*, 2004). A concentration as low as 0.3 nl l^{-1} is needed for both 8-carbon chain, 1-octylcyclopropene (1-OCP), and 10-carbon, 1-decylcyclopropene (1-DCP), to protect against ethylene response in banana fruits. Furthermore, the time of protection for 1-substituted CPs at ambient temperature (22-23°C) is significantly longer for 1-DCP (36 days) than that of 1-MCP (12 days) (Sisler *et al.*, 2003). Further 1-substituted CP tests have been done on kalanchöe and sweet pea flowers, but their inhibiting effects are lower than in bananas (Kebenei *et al.*, 2003a, b).

The effects of these compounds as ethylene antagonists are attributed to their structural molecular strain that permits a very tight binding to electron donor compounds such as low valency, in the receptor. They compete with ethylene for the binding sites and remain bound to the receptor for a long time, thus preventing ethylene from binding (Sisler *et al.*, 1999). The activity of CPs depends on the concentration required for inactivation of the receptor, with the duration of binding possibly due to steric and inductive effects, regarding the position of a methyl group with respect to double bonds (Sisler *et al.*, 1996a, b, 1999, 2001). Ethylene receptor inhibitors, such as 1-MCP, act by binding to a metal in the receptor. They compete with ethylene for the receptor, but do not induce ethylene responses (Sisler *et al.*, 1990).

Ethylene mediated flower senescence is a significant problem in horticulture, most obvious in the floriculture industry. The regulation of flower senescence is of great interest to horticulturists in search of methods to improve the postharvest quality of ornamentals. The need for chemical protection from ethylene action has been recommended in many potted flowering plants in which low concentrations of ethylene causes rapid loss in display quality (Serek, 1993; Serek *et al.*, 1994b, c). Therefore, much research has been conducted in order to develop anti-ethylene agents that block the synthesis and action of ethylene, as mentioned before. It is proposed that the analogues inhibit ethylene action by competing for the binding sites on the ethylene receptor, similar to the mode of action suggested for 1-MCP (Feng *et al.*, 2004). It is hypothesized that

1-OCP and 1-DCP, CPs with 8 and 10-carbon chain length, respectively, could significantly improve the display quality of miniature roses, important commercial potted plants, in protecting them against ethylene. This study reported the difference in the display quality between the two cultivars 'Lavender' with short flower life and 'Vanilla' with long-lasting flower, after pretreatment with 1-OCP, 1-DCP and 1-MCP, and afterwards continuous exposure to ethylene. The influence of treatment conditions, such as concentration, exposure time and temperature, on the postharvest quality of miniature rose plants was then investigated.
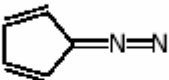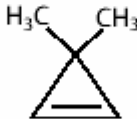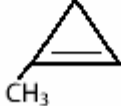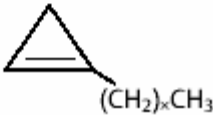
Compound	Structure
2,5-Norbornadiene (2,5-NBD)	
Trans-cyclooctene	
Diazocyclopentadiene (DACP)	=N=N
Cyclopropene (CP)	
3,3-Dimethylcyclopropene (3,3-DMCP)	H_3C CH_3
1-Methylcyclopropene (1-MCP)	CH_3
1-substituted cyclopropenes	$(CH_2)_xCH_3$

Fig. 3. Chemical structures of compounds interacting with the ethylene receptor (Sisler and Serek, 2003).

2.2 Materials and methods

2.2.1 Chemicals

1-OCP and 1-DCP were synthesized at the Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, USA, from 2-bromoalkenes and bromoform using 50% NaOH to produce a carbine and form a 1,1,2-cyclopropane, which was then reacted with methyllithium at dry ice temperature, to form cyclopropenes (Al Dulayymi *et al*., 1996, 1997). These compounds were then sent on dry ice to the University of Hannover, Germany. The compounds were subsequently divided into smaller samples of 0.5 ml and kept at -80°C until needed for the experiments. Each sample was then diluted with ether to a volume of 50 ml before being used for experiments. 1-MCP was obtained from AgroFresh Inc. (Rohm and Haas, AgroFresh Inc., Philadelphia, USA) in a commercially-available form.

2.2.2 Plant material

Rosa hybrida L. cultivar 'Lavender', which is sensitive to ethylene, was used to investigate the effects of extended chain length on the activity of inhibitors of ethylene receptors, 1-OCP and 1-DCP as compared with 1-MCP. In addition, miniature potted rose cultivar 'Vanilla', with excellent postharvest performance and low sensitivity to exogenous ethylene (Müller *et al*., 2001), was compared to 'Lavender' in the effects of exogenous ethylene after pretreatment with inhibitors of ethylene receptors (1-OCP and 1-DCP). 'Lavender', obtained from Kordana breeding line of Rosen Kordes, W. Kordes' Söhne Rosenschulen GmbH & Co KG, Sparrishoop, Germany, was used to investigate the effect of 1-OCP concentration on display quality of miniature potted roses. The other miniature potted roses were produced in 10 cm-diameter pots (2 cuttings per pot) in a greenhouse at the University of Hannover during January-December, under the following conditions: 19–20°C/20°C (day/night temperature) and 60-85% relative humidity (RH), natural daylight was supplemented with 60 μmol m^{-2} s^{-1} from SON-T lamps (Osram, 400W, Philips, Eindhoven, The Netherlands) over a 16 h (7.00-23.00) photoperiod. Paclobutrazol was used as a growth retardant at a concentration of 0.5%. Watering, fertilizer application and pest and disease control were carried out until plants were required for experiments. Miniature potted roses were used in the experiments when two to three flowers per pot had opened.

2.2.3 Effects of concentration of 1-OCP and 1-DCP on display quality

'Lavender' plants were placed in 54 l glass chambers. The plants were exposed to the desired concentrations of 1-OCP or 1-DCP (200, 500, 1000 and 1500 nl l^{-1}), respectively, and 200 nl l^{-1} of 1-MCP (Serek and Reid, 2000), sealed in glass chambers for 6 h at 20^oC. The calculated volumes of the 1-OCP and 1-DCP were pipetted on filter paper in the glass chambers to increase the surface area and facilitate evaporation. 1-MCP was released from a commercial powdered formulation (SmartFresh™, 0.14% a.i.) via addition of a few drops of water (≈0.5 ml). Control plants were sealed in identical containers containing air for 6 h at 20^oC. After the treatments, the chambers were vented for 30 min and the plants were transferred and placed randomly in a glass chamber, sealed and kept in an interior environment room, which was maintained at 20^oC, 60-70% RH. The plants were exposed to 12 h light from cool white fluorescent tubular lamps providing 20 µmol m^{-2} s^{-1}. The glass chambers were ventilated (200 l h^{-1}) with air containing a range of ethylene concentration of 1.25 (+/- 0.25) µl l^{-1} provided by a simple diffusion system (Saltveit, 1978) throughout the experimental period. The concentration of ethylene was determined daily using a Perkin-Elmer portable digital gas chromatograph (GC Voyager FFKG312, Ontario, Canada) equipped with a photoionisation detector. The carrier gas was N_2 at 40 ml min^{-1}, and the injection temperature was 60^oC with a column temperature of 60^oC. The display quality was determined after every 3 days by recording the percentage of leaf drop (80% leaf drop was considered unacceptable), and the number of days to onset of total bud drop (number of abscised or dead buds) and flower drop (individual flower longevity from the day of anthesis to petal drying or abscission).

2.2.4 Effects of treatment time on 1-OCP and 1-DCP on display quality

The optimum concentrations from section 2.2.3 were used. Potted 'Lavender' plants were selected and placed in 54 l glass chambers. They were treated with 1000 nl l^{-1} 1-OCP or 1-DCP in sealed glass chambers at 20^oC but at different exposure times (2, 4, 6 and 12 h). The 1-MCP treatments (200 nl l^{-1}) were sealed for 6 h (Serek *et al.*, 1994b, c; Serek and Reid, 2000b). Control plants were sealed in identical containers containing air for 6 h at 20^oC. After treatments, the plants were exposed to the same procedure as previously described in section 2.2.3.

2.2.5 Effects of temperature on efficacy of 1-OCP and 1-DCP on display quality

The temperature inside the growth chambers was set at 5, 10, 15 and 20°C and allowed to stabilize overnight. Potted 'Lavender' plants were selected and placed in 54 l glass chambers. They were treated with 1000 nl l^{-1} 1-OCP and 1-DCP in sealed glass chambers at 20°C but at different exposure times (2, 4, 6 and 12 h). 1-MCP treatments (200 nl l^{-1}) were sealed for 6 h (Serek *et al*., 1994b, c; Serek and Reid, 2000b). Control plants were sealed in identical containers containing air for 6 h at 20°C. After treatments, the plants were handled as previously described in section 2.2.3.

2.2.6 Comparison of effectiveness of 1-MCP, 1-OCP and 1-DCP between 'Lavender' and 'Vanilla' cultivars

Potted 'Lavender' and 'Vanilla' plants were selected and placed in 54 l glass chambers. They were treated with 1-OCP and 1-DCP at the best condition from the previous experiments in sealed glass chambers at 20°C. 1-MCP treatments (200 nl l^{-1}) were sealed for 6 h (Serek *et al*., 1994b, c; Serek and Reid, 2000b). Control plants were sealed in identical containers containing air for 6 h at 20°C. After treatments, the plants were exposed to the same procedure as previously described in section 2.2.3. During the experimental period, 1-OCP and 1-DCP experiments were conducted separately and 1-MCP was used in both experiments as the standard.

2.2.7 Experimental design and statistics

The experiments were conducted in a completely randomized design using 3 pots per treatment and two replications. Each pot contained 2 miniature rose cuttings. The data obtained was subjected to analysis of variance (ANOVA) using the general linear models (Proc GLM) procedure of the Statistical Analysis System (SAS, 2002) program package. Multiple comparisons among means was done using the Least Significant Difference (LSD) at P = 0.05.

2.3 Results

2.3.1 Effect of concentrations of 1-OCP and 1-DCP on display quality

Display quality was characterized by leaf flower and bud drop. In 'Lavender' the most obvious symptom of ethylene sensitivity was accelerated leaf drop. Miniature rose plants treated with a concentration of 200 nl l^{-1} 1-OCP and 1-DCP reached 80% leaf drop after 9 days of continuous exposure to ethylene and were not different from untreated (control) plants (Fig. 4A, B). Plants pretreated with 500-1500 nl l^{-1} of 1-OCP or 1-DCP exhibited reduced leaf drop and therefore improved display quality up to 15 days as compared to the control (Fig. 4A, B). Pretreatment with 1-MCP, 500-1500 nl l^{-1} 1-OCP, and all used concentrations of 1-DCP significantly ($P<0.001$) increased the number of days to the total bud and flower drop compared to control plants. There were no differences between 1-MCP and long chains CPs (Fig. 4C, D). The best display quality was achieved by pretreating miniature roses with 1000, 1500 nl l^{-1} for both 1-OCP and 1-DCP (Fig. 4A, B, C, D). Increasing the concentration of 1-OCP and 1-DCP from 1000 to 1500 nl l^{-1} did not show further improvement in the display quality of the plants. Also, no toxicity was observed with a high concentration of 1500 nl l^{-1}. Additionally, ether used to dissolve 1-OCP and 1-DCP had no effect on the display quality of 'Lavender' (data not shown). In terms of effectiveness of different concentrations, 1-MCP was more effective than 1-OCP and 1-DCP.
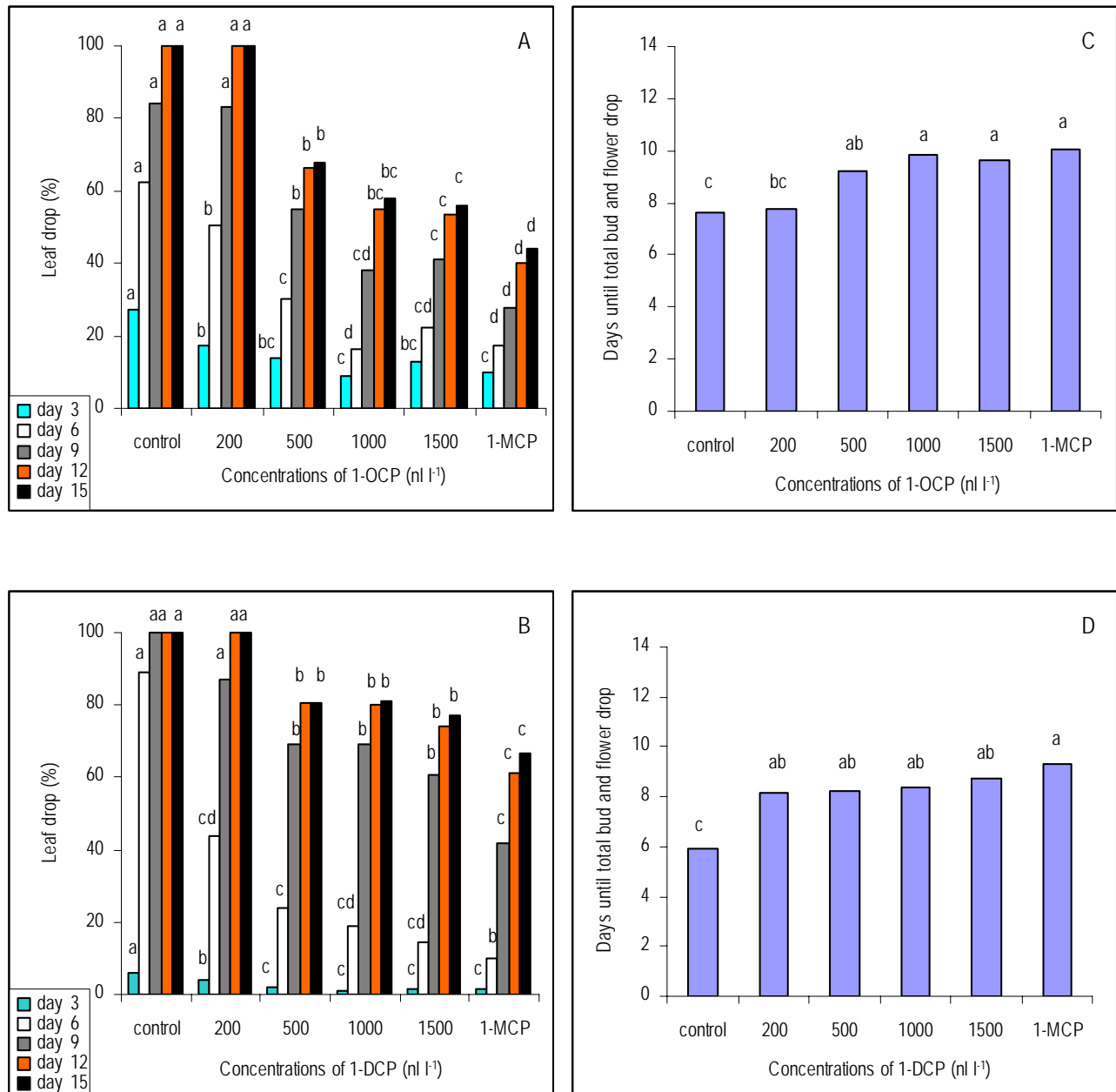
Fig. 4. Mean of percent leaf drop (A, B) and total bud and flower drop (C, D) of miniature potted rose cultivar 'Lavender' pretreated with 1-OCP and 1-DCP at concentrations of 0, 200, 500, 1000, 1500 nl l^{-1} and untreated controls, respectively. These were compared to treatment with 1-MCP (200 nl l^{-1}) for 6 h at 20°C. After the treatments, the plants were exposed to 1.25 (+/- 0.25) µl l^{-1} continuous ethylene throughout the experiments. Bars marked with the same letter (for figures A, B, C, D bars representing the same day) are not statistically different at *P<0.05*. Means were separated by LSD.

2.3.2 Effect of exposure time of 1-OCP and 1-DCP on display quality

Exposing miniature potted roses to 1000 nl l^{-1} 1-OCP or 1-DCP for 4 to 12 h significantly (P<0.001) delayed leaf drop (Fig. 5A, B) and increased the number of days to the onset of total bud and flower drop (Fig. 5C, D). However, an exposure time of 2 h had no effect on leaf drop or total bud and flower drop. Plants pretreated for a short time (2 h) showed a fast response to ethylene by reaching 80% leaf drop after day 9 (Fig. 5A, B) and took about 7 days to reach the total bud and flower drop for both 1-OCP and 1-DCP (Fig. 5C, D). The untreated (control) plants reached 80% leaf drop within 6 days as well as the total bud and flower drop, respectively (Fig. 5A, B, C, D). Within the time range (4-12 h), 1-OCP treatments were less effective than 1-MCP, with respect to leaf drop. It also took 1-OCP treated plants 8 days to attain the total bud and flower drop while 1-MCP-treated plants took 12 days. The 1-DCP treatments were not different from 1-MCP with respect to percent leaf drop. Exposing plants to 1000 nl l^{-1} 1-DCP for 2 to 12 h significantly increased the number of days to total bud and flower drop (Fig. 5D). Additionally, after 12 h exposure to 1-DCP, it took 10 days to attain total bud and flower drop. However, 1-MCP was the most effective in preventing bud and flower drop.
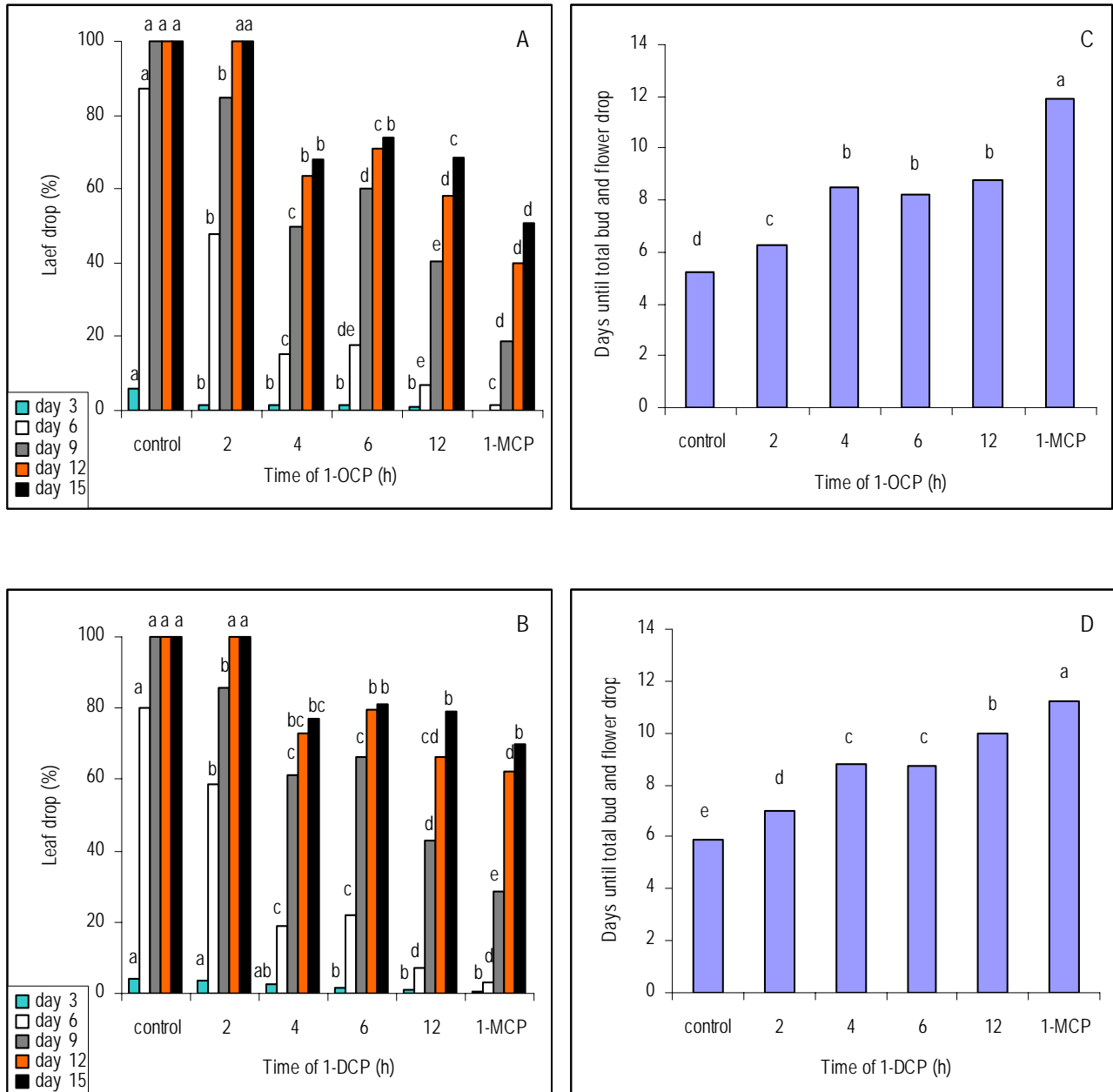
Fig. 5. Mean of percent leaf drop (A, B) and total bud and flower drop (C, D) of miniature potted rose cultivar 'Lavender' pretreated with 1-OCP and 1-DCP (1000 nl l^{-1}) at exposure time of 0, 2, 4, 6, 12 h and untreated controls, respectively. These were compared to treatment with 1-MCP (200 nl l^{-1}) for 6 h at 20°C. After the treatments, the plants were exposed to 1.25 (+/- 0.25) µl l^{-1} continuous ethylene throughout the experiments. Bars marked with the same letter (for figures A, B, C, D bars representing the same day) are not statistically different at *P<0.05*. Means were separated by LSD.

2.3.3 Effect of temperature of 1-OCP and 1-DCP on display quality

Treatment of miniature roses with 1-OCP and 1-DCP (1000 nl l^{-1}) for 4 h at all temperature regimes (5, 10, 15 and 20°C) significantly improved their display quality as showed by reduced leaf drop, total bud and flower drop compared with the control (Fig. 6A, B, C, D). All temperature levels significantly ($P<0.001$) decreased leaf drop and increased number of days to the onset of total bud and flower drop as compared to the untreated (control) plants (Fig. 6A, B, C, D). No significant difference was observed in display life among all the temperature levels (5 to 20°C) in flowers treated with 1-OCP and 1-DCP. Plants treated with 1-MCP gave better results than that of 1-OCP and 1-DCP.
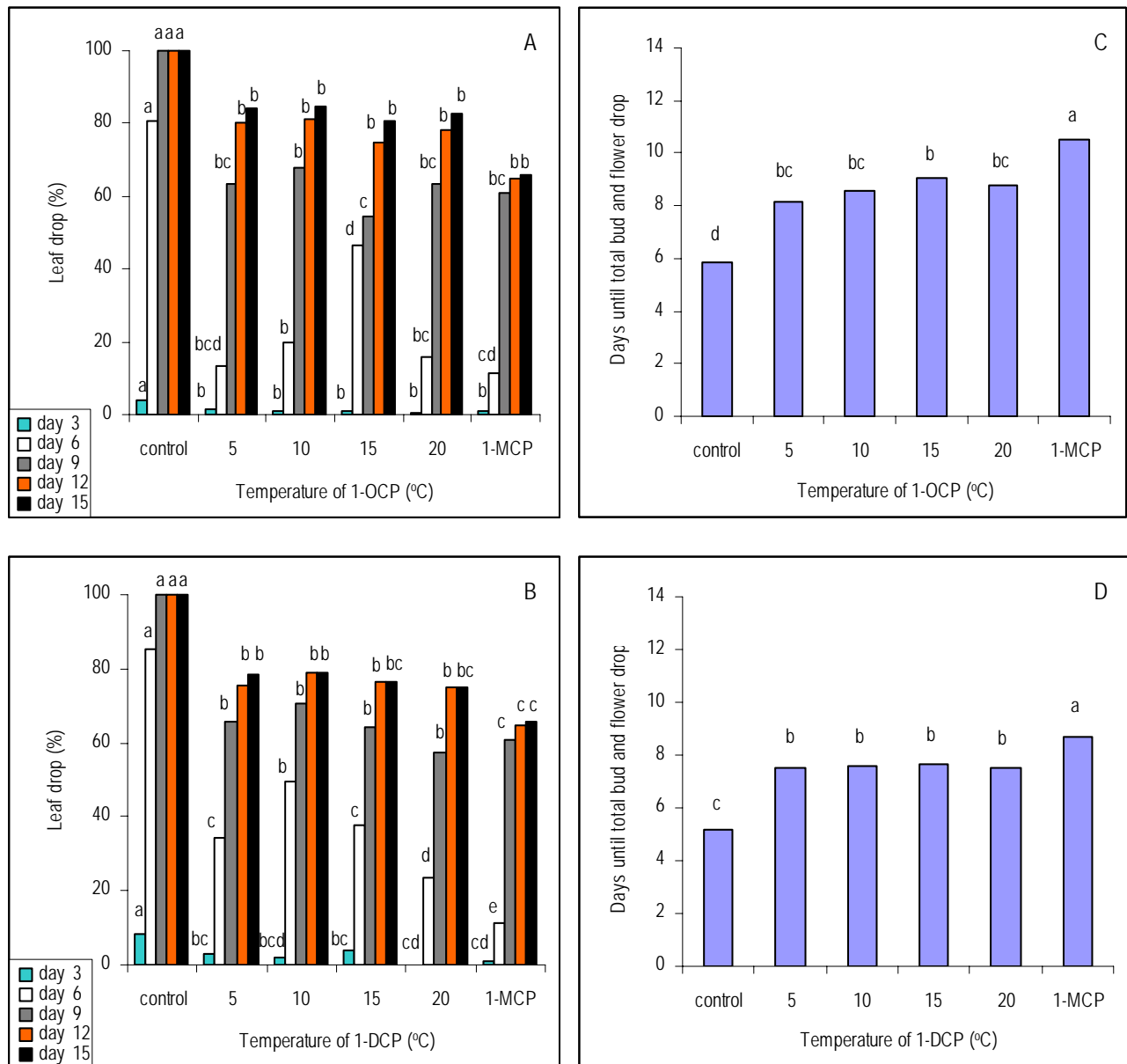
Fig. 6. Mean of percent leaf drop (A, B) and total bud and flower drop (C, D) of miniature potted rose cultivar 'Lavender' pretreated with 1-OCP and 1-DCP at temperatures of 5°C, 10°C, 15°C, 20°C, and untreated controls, respectively. These were compared to treatment with 1-MCP (200 nl l^{-1}) for 6 h at 20°C and untreated controls. After the treatments, the plants were exposed to 1.25 (+/- 0.25) µl l^{-1} continuous ethylene throughout the experiments. Bars marked with the same letter (for figures A, B, C, D bars representing the same day) are not statistically different at *P<0.05*. Means were separated by LSD.

2.3.4 Efficacy of 1-MCP, 1-OCP and 1-DCP in cultivars 'Lavender' and 'Vanilla'

Efficacy of ethylene action inhibitors 1-MCP, 1-OCP and 1-DCP between 'Vanilla' (long lasting flower) and 'Lavender' (short flower life) was investigated. In both cultivars, ethylene accelerated flower senescence. However, there were distinct differences between 'Vanilla' and 'Lavender' when they were continuously exposed to 1.25 μl^{-1} ethylene (Fig. 7A, B, C, D). Untreated 'Vanilla' plants attained 40% leaf drop after 6 days of continuous exposure to ethylene, while the display quality of 'Lavender' was clearly reduced to 80% leaf drop in the same period (Fig. 7A, B). Additionally, it took 6 days to attain total bud and flower drop in 'Lavender' (Fig. 7D). Pretreatment with ethylene receptor inhibitors 1-OCP, 1-DCP and 1-MCP, improved the display quality in both cultivars. In 'Vanilla', the trend of percent leaf drop in flowers treated with 1-OCP and 1-DCP was similar to that of 'Lavender'. Also, 1-OCP-treated plants reached 100% leaf drop after day 15 and were less effective than 1-DCP (Fig. 7A, B). There was no significant difference between the display quality of 1-DCP and 1-MCP. However, it took 9 and 10 days for 'Vanilla' plants to attain total bud and flower drop for 1-OCP and untreated (control) plants, respectively. It took 11 and 12 days for 'Vanilla' plants treated with 1-DCP and 1-MCP to attain the total bud and flower drop (Fig. 7C), respectively. In 'Lavender', 1-OCP compared favorably with 1-DCP with respect to percent leaf drop, while pretreatment with 1-MCP was the most effective in improving the display quality of miniature roses. The untreated 'Lavender' plants took 6 days to attain total bud and flower drop while it took 8, 9 and 10 days for 1-OCP, 1-DCP and 1-MCP, respectively (Fig. 7D).
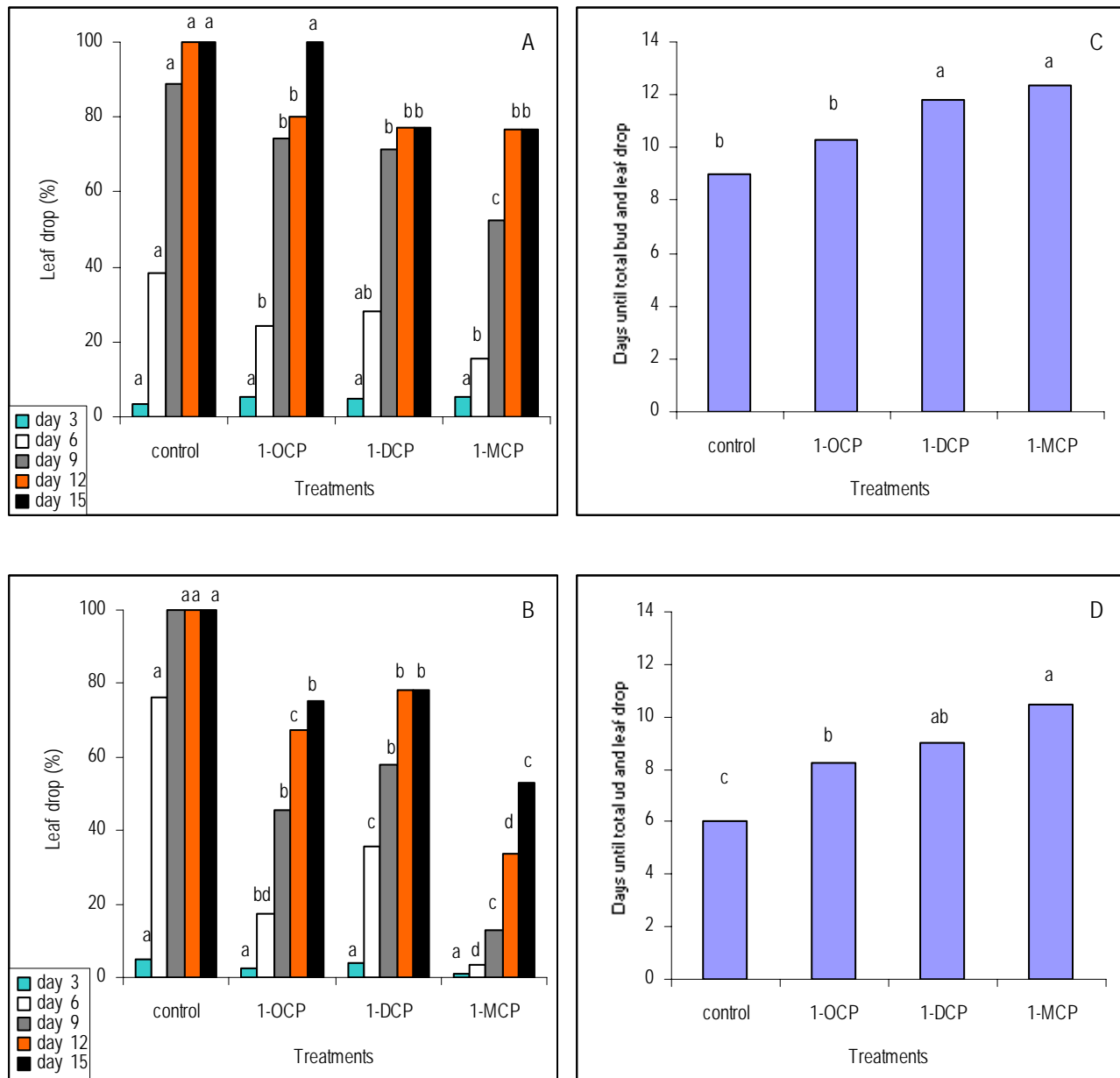
Fig. 7. Mean of percent leaf drop and days of total bud and flower drop of miniature potted rose cultivars 'Vanilla' (A, C) and 'Lavender' (B, D) pretreated with 1-OCP, 1-DCP (1000 nl l^{-1}), respectively, 1-MCP (200 nl l^{-1}) for 6 h at 20°C and the control. After the treatments, the plants were exposed to 1.25 (+/- 0.25) µl l^{-1} continuous ethylene throughout the experiments. Bars marked with the same letter (for figures A, B, C, D bars representing the same day) are not statistically different at *P<0.05*. Means were separated by LSD.

2.4 Discussion

The duration of quality improvement was dependent on the concentrations. 1-OCP and 1-DCP, two structural analogues of 1-MCP with substitution in 1-position, were considered to be putative inhibitors of ethylene action. Their potency was evaluated by their ability to counteract ethylene-induced responses (leaf, bud and flower abscission). These two analogues exerted their effect in a similar manner to 1-MCP by blocking the binding site of ethylene in the receptor (Sisler and Serek, 1997; Feng *et al.*, 2004). In this study, all of these compounds were effective in blocking the ethylene receptor and improving the display quality of miniature potted roses when compared to untreated control plants. Based on the substitution with 8 and 10 carbon chains at 1-position of the cyclopropene, both compounds were effective in improving display quality from exogenous ethylene, but in terms of concentration, they were less effective than 1-MCP. Moreover, the concentration required for 1-OCP and 1-DCP was about 5 times higher than that of 1-MCP. This is in contrast to the previous studies of Sisler *et al.*, (2003) and Kebenei *et al.*, (2003a, b), where 1-OCP was effective at lower concentrations than 1-MCP in delay ripening in bananas and improving display life and quality of kalanchöe and sweet pea, respectively. Sisler and Serek (1997) suggested that for the maximum response in growing vegetative tissue, a higher concentration of an inhibitor is needed. Besides, flowers on intact plants, as used by Müller *et al.* (2000a, 2002b), may react differently to exogenous ethylene treatments than detached kalanchöe flowers (Kebenei *et al.*, 2003a, b). Probably the presence of many leaves in whole plants could have absorbed higher amounts ethylene than excised flowers (Müller and Stummann, 2003). In miniature roses, pretreatment with 1-MCP obviously reduced the rise in autocatalytic ethylene production in flowers (Müller and Stummann, 2003), whereas in vegetative tissue, such as tobacco leaves, ethylene production was autoinhibitited by a negative feedback control (Philosoph-Hadas *et al.*, 1985). Furthermore, another factor that should be taken into consideration is the time it takes ethylene inhibitors to bind to the receptors and regain activity. This suggests that the same receptor is becoming active again or that new receptors are being produced (Sisler *et al.*, 1990). The fact that some time after the treatment with 1-OCP or 1-DCP the tissue became sensitive to ethylene again, assumes that free binding sites on the ethylene receptor are present in the tissue at the point of recovery from the inhibition. However, the analogue 1-OCP was found to be a more potent inhibitor than 1-DCP, when pretreating ethylene-

sensitive cultivars like 'Lavender'. In contrast, pre-treating 'Vanilla', which has long-lasting flowers, with 1-DCP was more effective than 1-OCP.

Both 1-OCP and 1-DCP are gases at room temperature (Closs, 1966). They need to be dispersed as liquids that need time to evaporate and diffuse to the binding sites in order to bind to them. Display quality was dependent on exposure time, such that longer exposure times improved the display quality better than shorter exposure times. Similar observations were reported on other cyclopropenes (Dupille and Sisler, 1995; Sisler et al., 1996a, 1999). Molecular weight has no influence on evaporation, in that higher molecular weight compounds bind as rapidly as the lower ones, suggesting evaporation time into the gaseous phase is not a big factor (Sisler et al., 2003). Continuous exposure for longer periods results in saturation of the receptor and further treatment does not give any additional effect. For example, exposure of both chemicals for 12 h gave the same effect as 4 h exposure. Therefore, a 4-h exposure is sufficient to give better improvement against ethylene. Shorter exposure time (2 h) with the same concentration was not satisfactory. This may require a higher concentration of the respective cyclopropenes (Sisler et al., 1999, 2003) for the same beneficial effect to be realized.

Temperature has a direct influence on the exposure time and concentration of the treatment. At lower temperatures, a higher concentration and longer time of treatment is required (Sisler et al., 1996a, 1999; Sisler and Serek, 1997). In this study, the temperature range 5-20°C had no effect on the activity of both chemicals, implying that pre-treatment of potted roses with these compounds can be done within temperatures that range from 5-20°C without any affects on their effectiveness. Contrary to kalanchöe (Kebenei et al., 2003a, b), the activity of 1-OCP was influenced by temperature, and it was more potent at 15 and 20°C than at lower (5 and 10°C) temperatures. In carnations and Penstemon, a higher concentration of 1-MCP was required at low temperatures in order to give protection against ethylene (Serek et al., 1995a; Sisler et al., 1996a).

Differences in ethylene production and/or sensitivity are a vital factor regulating flower life. For this reason ethylene partly resulted in differential display quality (Müller et al., 1998, 2001). Ethylene caused leaf, bud and flower drop, but there were differences among cultivars. 'Lavender', which is sensitive to ethylene, significantly exhibited 80% leaf drop as well as a total bud and flower drop within 6 days as compared to 'Vanilla', which took 9 days. It could be

assumed that 'Lavender' was similar to cultivar 'Bronze', in which exposure to ethylene induces an autocatalytic rise in ethylene with constant increase in endogenous ethylene levels (Müller et al., 2001). Although 'Vanilla' had excellent longevity and seemed to be almost completely insensitive to exogenous ethylene (Müller et al., 2001), continuous exposure to ethylene caused flower, bud and leaf drop. In the current study, 'Vanilla' had about 80% leaf and total bud drop within 9 days, which was retarded by ethylene inhibitors. In the miniature rose 'Victory Parade', 1-MCP protected against exogenous ethylene, increased display life and reduced abscission of buds, flowers and leaves (Serek et al., 1994c). Therefore, for ethylene-sensitive cultivars like 'Lavender', ethylene inhibitor treatment will be an important practical tool in increasing the postharvest life after ethylene exposure. It would also be more effective in improving the display quality of the ethylene-sensitive cultivar 'Lavender' than ethylene-insensitive 'Vanilla'.

In conclusion, these results demonstrated the potency of 1-MCP and long chain CPs, 1-OCP and 1-DCP, as ethylene antagonists in improving display quality of miniature potted roses. Additionally, 1-MCP was the most potent ethylene inhibitor in terms of concentration and duration of exposure in improving display quality as compared to 1-OCP and 1-DCP. Therefore, 1-MCP is more suitable for use in potted plants while 1-OCP is more effective in prolonging the display life of individual flowers like kalanchöe.

3. Expression analysis of genes for ethylene biosynthesis enzyme, ethylene perception and signal transduction pathway after pretreatment with ethylene receptor inhibitors of miniature potted roses (*Rosa hybrida* L.)

Abstract

In order to investigate transcript abundance of genes for ethylene biosynthesis enzyme (*RhACS1-5*), ethylene perception (*RhETR1-4*) and signal transduction pathway (*RhCTR1-2, RhEIN3, RhEIL*), expression of these transcripts in petal and leaf tissues was examined in two rose cultivars 'Vanilla' and 'Lavender' during continuous exposure to exogenous ethylene and ethylene-free air after pretreatment with inhibitors of ethylene receptors 1-MCP, 1-OCP and 1-DCP. Additionally, one of the ethylene-responsive element-binding factor (*ERFs*) genes was isolated from miniature potted roses (*RhERF1*) which was 92 bp long, encoding a predicted polypeptide for an Ap2 DNA binding domain and sharing high homology with other reported ERF domains. Northern blot hybridization analysis was used to compare with results of RT-PCR. However, it was difficult to distinguish the hybridization signal of ethylene receptors between *RhETR1* and *RhETR2*, which were highly homologue. RT-PCR analysis revealed that ethylene induced the expression of all genes investigated in control 'Vanilla' and 'Lavender' petals except *RhACS1-2* and *RhETR4*, respectively, while pretreatments of 1-MCP and 1-OCP led to the supression of all investigated mRNAs in the presence or absence of ethylene. However, strong expression of *RhETR3, RhEIN3* and *RhEIL* transcripts was detectable in 'Lavender' petals pretreated with 1-DCP. In the absence of ethylene, strong expression of all genes was detectable in control 'Vanilla' leaves but there was no expression of *RhACS1*, while the accumulation of all genes was eliminated after pretreatments of 'Vanilla' leaves with ethylene receptor inhibitors. The level of *RhEIN3* mRNA was upregulated by pretreatment of 1-MCP, 1-OCP and 1-DCP in 'Vanilla' leaves in the presence of ethylene, while the accumulation of *RhEIN3* transcript was suppressed in 'Lavender' leaves after pretreatment of 1-MCP and 1-OCP. *RhEIN3* transcript is probably rate-limiting for ethylene perception and signal transduction pathway, which is regulated in leaves during flower senescence at the transcriptional level. In the absence of ethylene, 1-MCP suppressed the expression of *RhACS1, RhETR3* and *RhERF1* transcripts. Similarly, 1-OCP inhibited the expression of *RhACS1, RhCTR2* and *RhERF1* transcripts. 1-DCP inhibited the expression of

RhEIN3, *RhEIL* genes. However, in the presence of ethylene, pretreatment of 'Lavender' leaves with 1-MCP suppressed the expression of *RhACS1*, *RhETR4*, *RhCTR1-2*, *RhEIL* and *RhERF1* transcripts. Similarly, 1-OCP treatment inhibited the expression of *RhACS1*, *RhETR2*, *RhETR4*, *RhCTR1-2*, *RhEIN3* and *RhERF1* transcripts. Pretreating 'Lavender' leaves with 1-DCP suppressed the expression of *RhACS1* and *RhETR4* transcripts. These results indicated that the expression of genes for the ethylene biosynthesis enzyme, ethylene perception and signal transduction pathway was regulated by both positive and negative feedback regulation mechanisms in 'Vanilla' and Lavender' pretreated with ethylene receptor inhibitors in the presence or absence of ethylene. This might depend on plant species and tissues under investigation. Physiologically and molecularly, these results suggest that pretreatments with inhibitors of ethylene receptors improved the display life of miniature roses by delaying bud, flower and leaf drop. However, 1-MCP is more effective than 1-OCP and 1-DCP by suppressing the expression of ethylene signal transduction genes or the feedback regulations of genes for ethylene biosynthesis and ethylene perception.

Key words: ACC synthase, display quality, ethylene, ethylene perception and signal transduction, gene expression, inhibitors of ethylene receptor, *Rosa hybrida,* senescence

3.1 Introduction

3.1.1 Expression regulation of genes for ethylene biosynthesis enzyme

Ethylene biosynthesis and perception are modulated during development of plant tissues (Abeles *et al.*, 1992) and responsible for inducing many biochemical processes which lead to programmed cell death, including the expression of senescence-related genes (Woodsons *et al.*, 1992; Kim *et al.*, 1997). Ethylene biosynthesis in higher plants has been well characterized and 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (*ACS*), an enzyme of ethylene biosynthesis, has been recognized as the rate-limiting step (Yang and Hoffman, 1984; Kende, 1993).

In all plants, *ACC* synthase is encoded by a medium-size gene family, which is differentially regulated in a tissue-specific manner by a variety of developmental cues, mechanical and environmental stresses and by ethylene itself (Kende, 1993; Zarembinski and Theologis, 1994). In tomato fruits, ACC synthase is a soluble enzyme (Boller *et al.*, 1979). This indicates that ACC synthase is a cytoplasmic enzyme (Kende, 1993).

Sequence analysis of the *Arabidopsis* genome reveals that *ACS* genes are putatively encoded by twelve genes (Yamagami *et al.*, 2003). All genes, except *ACS3*, are transcriptionally active and differentially expressed in *Arabidopsis* during growth and development. Additionally, IAA induces all genes, except *ACS7* and *ACS9*; cyclohexamide (CHI) enhances the expression of all functional *ACS* genes. These *ACS* genes, which have a biochemically-diverse function in unique cellular environments for the biosynthesis of ethylene, permit the signaling molecule to exert their unique effects in a tissue- or cell-specific manner (Yamagami *et al.*, 2003).

In *Rosa hybrida*, *ACS* genes consist of a multigene family (Mibus and Serek, 2004; Wang *et al.*, 2004). Analysis of the expression of *ACS* genes shows that *RhACS1* is specifically expressed in rose petals, the ovary and sepals. This expression increases dramatically as the flower matures to senescence and also correlates positively with ethylene levels (Wang *et al.*, 2004).

3.1.2 Ethylene receptor gene expression

Ethylene actions are involved with binding to a receptor, followed by activation of one or more signal transduction pathways that lead to the cellular response. Ultimately, ethylene exerts its effects primarily by changing the pattern of gene expression (Taiz and Zeiger, 2002). Furthermore, the development of molecular genetic approaches in the model plant *Arabidopsis* has established the linear signal transduction pathway for the response to ethylene (Hongwei and Ecker, 2004). Genetic screens based on the triple-response phenotype, inhibition of hypocotyls and root cell elongation, radial swelling of hypocotyls and exaggerated curvature of the apical hook, have been extensively conducted on etiolated *Arabidopsis* seedlings to isolate mutants that are affected in their response to ethylene (Bleecker *et al.*, 1988; Guzman and Ecker, 1990). Two classes of mutants have been identified:

1. Mutants that fail to respond to exogenous ethylene (ethylene-resistant or ethylene-insensitive mutants).
2. Mutants that display the response even in the absence of ethylene (constitutive mutants).

Ethylene-insensitive mutants are identified as tall seedlings extending above the lawn of short, triple-responding seedlings when grown in the presence of ethylene. Conversely, constitutive ethylene response mutants are identified as seedlings displaying the triple response in the absence of exogenous ethylene (Guzman and Ecker, 1990).

The *ETR1* gene is the first member of the receptor gene family to be cloned from *Arabidopsis* (Chang *et al.*, 1993). Expression of *ETR1* protein, which is obtained from plants and transgenic yeast, is not only membrane-associated but also exists as a covalently-linked dimer. Two cycteines at the N-terminus (Cys4 and Cys6) are the sites of disulphide cross-linkage between *ETR1* monomers. This N-terminal sensor domain also consists of all the elements essential and sufficient to bind ethylene with high affinity when expressed in yeast (Schaller *et al.*, 1995; Schaller and Bleecker, 1995). The C-terminal half of the *ETR1* protein contains all of the conserved sequence elements found in the histidine kinase domains of bacterial two-component regulators. These are typically composed of a sensor protein with an input domain receiving signals and a catalytic transmitter domain which autophosphorylates on an internal histidine

residue. The second component is a response regulator protein consisting of a receiver domain, which receives phosphate from the transmitter and an output domain that mediates responses depending on the phosphorylation state of the receiver. The *ETR1* protein has a receiver domain fused to the C-terminus of the histidine kinase domain (Parkinson, 1993; Maeda *et al.*, 1994). In addition, the two-component system is a signaling mechanism primarily used by bacteria to respond to a broad array of environmental signals (Stock *et al.*, 2000). However, the region that is located between the hydrophobic sensor domain and the histidine kinase transmitter is of unknown function. Besides, there are two blocks of sequence within this region, which shows homology to sequences that flank the chromophore-binding domain in the light-sensing phytochromes from higher plants and cyanobacteria (Kehoe and Grossman, 1996). It has been reported that the region between these blocks of homology in *ETR1* contains a GAF domain associated with cyclic GMP binding and light regulation in a number of proteins, but the function of the GAF domain in *ETR1* is unknown (Aravind and Ponting, 1997). Studies of *ETR1* indicate that localization of transmembrane domains on the receptor also serve to the endoplasmic reticulum (ER), an unusual location for a hormone receptor, but compatible with the ready diffusion of ethylene in aqueous and lipid environments (Chen *et al.*, 2002).
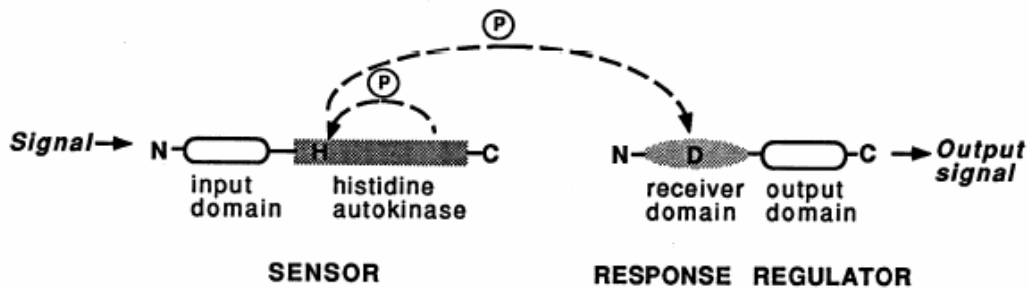
Fig. 8. Basic scheme of the two-component system (Chang and Meyerowitz, 1995).

Ethylene-binding domain of *ETR1* lies within the first 165 amino acids of the protein containing the sequences that are necessary and sufficient to bind ethylene. Further characterization of ethylene binding to *ETR1* occurs in a hydrophobic pocket, located at the N terminus of the receptor, and requires a transition metal, copper, as a cofactor (Schaller and Bleecker, 1995; Rodriguez *et al.*, 1999). The identification of a copper ion is associated with the ethylene-binding domain of *ETR1* with theoretical considerations dating back to Stanley Burg's original hypothesis

that ethylene binding would be mediated by a transition metal cofactor (Burg and Burg, 1967). Interestingly, silver (Ag(I)), which has long been known to inhibit ethylene-responses, may also occupy the binding site and interact with ethylene but fail to induce the changes that would normally occur following copper-mediated ethylene binding (Rodriguez *et al*., 1999). Cloning of *Arabidopsis RAN1* resembles copper transporting P-type ATPase and rescues a copper transport defect in yeast (Hirayama *et al*., 1998). Within the plant, *RAN1* potentially serves to produce functional ethylene receptor via intracellular delivery of copper. The loss-of-function of *ran1* mutants displays a rosette-lethal phenotype, which is thought to be caused either by general effects due to the reduced copper availability for other copper-utilizing enzymes, or by disruption of ethylene receptor function (Hirayama *et al*., 1998; Woeste and Kieber, 2000).
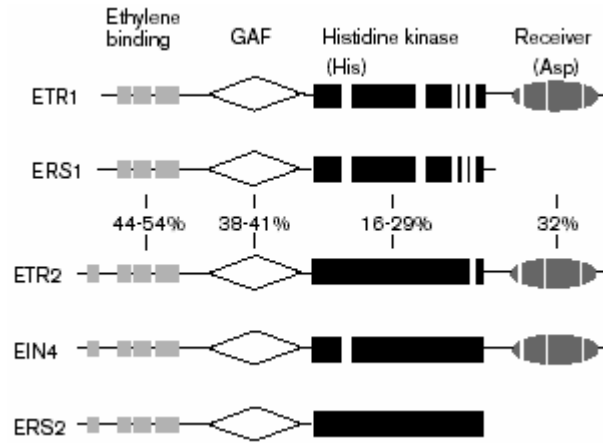
Fig. 9. The *Arabidopsis* ethylene receptor family (Chang and Shockey, 1999).

Five *ETR1-like* genes have been identified in *Arabidopsis* so far. These five genes can be divided into two main subfamilies based on sequence similarity and structure features of the protein. Members of type-I subfamily *ETR1* and *ERS1* (Hua *et al*., 1995, 1998), are most closely related in sequence and have three hydrophobic subdomains at the N-terminus. The type-II subfamily receptors, *ETR2* (Sakai *et al*., 1998), *ERS2* and *EIN4* (Hua *et al*., 1998), share close sequence similarity and all have four hydrophobic subdomains at the N-terminus.

Like *ETR1*, *ERS1* binds ethylene when expressed in yeast (Schaller *et al*., 1995), and its transmitter domain containing all the conserved subdomains (H, N, G1, F and G2) is thought to be required for histidine kinase activity. *ERS1* differs from *ETR1* in that it lacks the fused

receiver domain. Conversely, members of the *ETR2*-like subfamily appear to be degenerate in the histidine kinase domain. Each member of this subfamily lacks one or more elements that are thought to be essential for histidine kinase activity; therefore it is unlikely that they are functional kinases (Schaller *et al*., 1995).

In miniature potted roses, fragments of four ethylene receptor genes have been isolated and cloned, termed *RhETR1-4* (Müller *et al.,* 2000a, b). They are an ethylene receptor gene family with 2 subfamilies, as in *Arabidopsis*. *RhETR1* and *RhETR4* exhibit high amino acid similarity to *AtERS1*, while *RhETR2* is more similar to *AtETR1*. Three of the receptors belong to subfamily 1, whereas *RhETR3* belongs to subfamily 2. The northern blot analysis is conducted in miniature roses for *RhETR1-3* during flower senescence after treatment with ethylene and ABA to investigate the differences in flower longevity and ethylene sensitivity in miniature roses. The relative transcript abundance in the flowers varies significantly during development and after hormone treatment, but the expression of all three genes is detectable at all flower stages. Exposure to low ethylene concentrations results in an upregulation of *RhETR1* and *RhETR3* in flowers of 'Bronze', a short-life flower, and are distinctly higher than long-lasting 'Vanilla'. It is assumed that differences of cultivars in flower sensitivity to ethylene may partly be due to differences in receptor expression levels during flower development (Müller *et al*., 2000a, b).

While *RhETR2* expression varies slightly during flower development and in response to ethylene and ABA treatment, the expression of *RhETR1* and *RhETR3* exhibits differently during flower development and appears to be rate-limiting for ethylene perception and the determinants of flower longevity. It is found that the expression of *RhETR1* in 'Bronze' is distinctly higher than long-lasting 'Vanilla'. Although the expression of *RhETR1* precedes the ethylene production by the flowers, abundance of the *RhETR3* transcript increases during flower senescence in 'Bronze', indicating that the ethylene response system in rose flowers consists of multiple receptor genes with an overlapping pattern of expression. In 'Vanilla', a cultivar with excellent flower longevity despite moderate ethylene production (Müller *et al*., 2000b), expression of *RhETR1* and *RhETR3* is reduced. These results indicate that differences in flower life among rose cultivars – in an ethylene free environment and in response to exogenous ethylene – may be due to differences in receptor expression levels. These results do not conform to the standard model of ethylene signal transduction (Bleecker, 1999), which predicts that a reduction in the level of receptors would

result in increased ethylene sensitivity, while an increase in receptor numbers would result in decreased sensitivity.

3.1.3 Ethylene-insensitive mutant of ethylene receptor

Mutations in the ethylene receptors cause ethylene insensitivity or constitutive ethylene responses, dependent on the nature of the mutation. Ethylene insensitivity is a result of single amino acid changes within the region of the receptor involved in ethylene binding (Chang *et al*., 1993; Hua *et al*, 1995, 1998; Sakai *et al*, 1998). The ethylene-insensitive mutations are dominant. Little or no effect of single loss-of-function mutations upon ethylene signaling transduction is investigated by experiments using combinations of receptor knock-out (Hua and Meyerowitz, 1998), indicating that there is functional overlap among the receptor family members and the receptors serve as negative regulators of the ethylene response pathway, because elimination of receptors activates ethylene responses. According to this model for negative regulation, wild-type ethylene receptors actively repress ethylene responses in the air but express in the presence of ethylene. Wild type receptors switch to a signaling inactive state that allow for induction of ethylene responses (Bleecker, 1999). In contrast to the single and double loss-of-function, triple and quadruple receptor mutants show constitutive-ethylene response phenotypes because the basal levels of ethylene, which are constitutively produced in these plants, are sufficient to inactivate the remaining receptor 'repressive' activity (Cancel and Larsen, 2002). Therefore, an ethylene-insensitive mutation in one member of the five-member ethylene insensitivity suggests that signaling by one family member is enough to repress ethylene responses. Conversely, loss-of-function mutations in three receptors are simultaneously sufficient to induce ethylene responses (Hua and Meyerowitz, 1998). Ethylene bind to the receptor alters the activity of the His kinase domain, which in turn influences downstream components *CTR1* and *EIN2* (Guzman and Ecker, 1990; Kieber *et al*., 1993; Ecker, 1995).

Mutant, *etr1-1*, which no longer binds copper, is incapable of binding ethylene (Rodriguez *et al*., 1999), and the receptor is unable to turn *CTR1* "on" even when ethylene is present, resulting in the ethylene-insensitive phenotype. The expression of the *Arabidopsis etr1-1* gene in transgenic carnations delays flower senescence, which leads to a significant increase in vase life. About half of the transgenic plants obtain flower senescence, which delays a 3-fold increase in vase life by at

least 6 days relative to control flowers, with a maximum delay of 16 days. These flowers do not show the phenotype typical of ethylene-dependent carnation flower senescence (Bovy *et al.*, 1999). The expression of the *ACO1* gene in *etr1-1* transgenic carnation flowers is down regulated, suggesting that the autocatalytic induction of ethylene biosynthesis is absent due to dominant ethylene insensitivity. The delay in senescence observed in *etr1-1* transgenic flowers is longer than in flowers pretreated with inhibitors of either ethylene biosynthesis (AOA) or ethylene receptor (STS) (Van altvorst *et al.*, 1995, 1997; Bovy *et al.*, 1999). Agrobacterium-mediated transformation of tomato, tobacco and petunia plants with *Arabidopsis etr1-1* gene under control of the constitutive CaMV 35S promoter is successfully conferred ethylene insensitivity. An ethylene-insensitive phenotype delays flower senescence after pollination and after exogenous ethylene treatment (Wilkinson *et al.*, 1997). Ciardi and Klee (2001) elucidated mode of the dominant *etr1-1* mutation, which causes a substitution of tyrosine (Y65) for cysteine at amino acid position 65, eliminating both ethylene and copper binding (Fig. 10). Since ethylene does not bind, phosphorylation cannot be inactivated and the ethylene response is constitutively suppressed. Additionally, ethylene inhibitor action, STS and 1-MCP, associated with theoretical considerations (Sisler and Serek, 1997), can be explained at the molecular level that the receptor (*ETR1*) exhibits strong affinity for another metal, silver, and mediates ethylene binding. Silver probably acts by displacing copper in the active site of the receptor complex that still binds to ethylene (Ciardi and Klee, 2001). Therefore it must prevent a conformational change that would normally occur following copper-mediated ethylene binding. The compound 1-MCP, can also be bound by ethylene, but binding does not increase ethylene sensitivity and serves to block ethylene perception instead (Fig. 10). Although ethylene receptors are capable of binding several different molecules, the interaction required to induce an ethylene response is extremely specific (Ciardi and Klee, 2001).
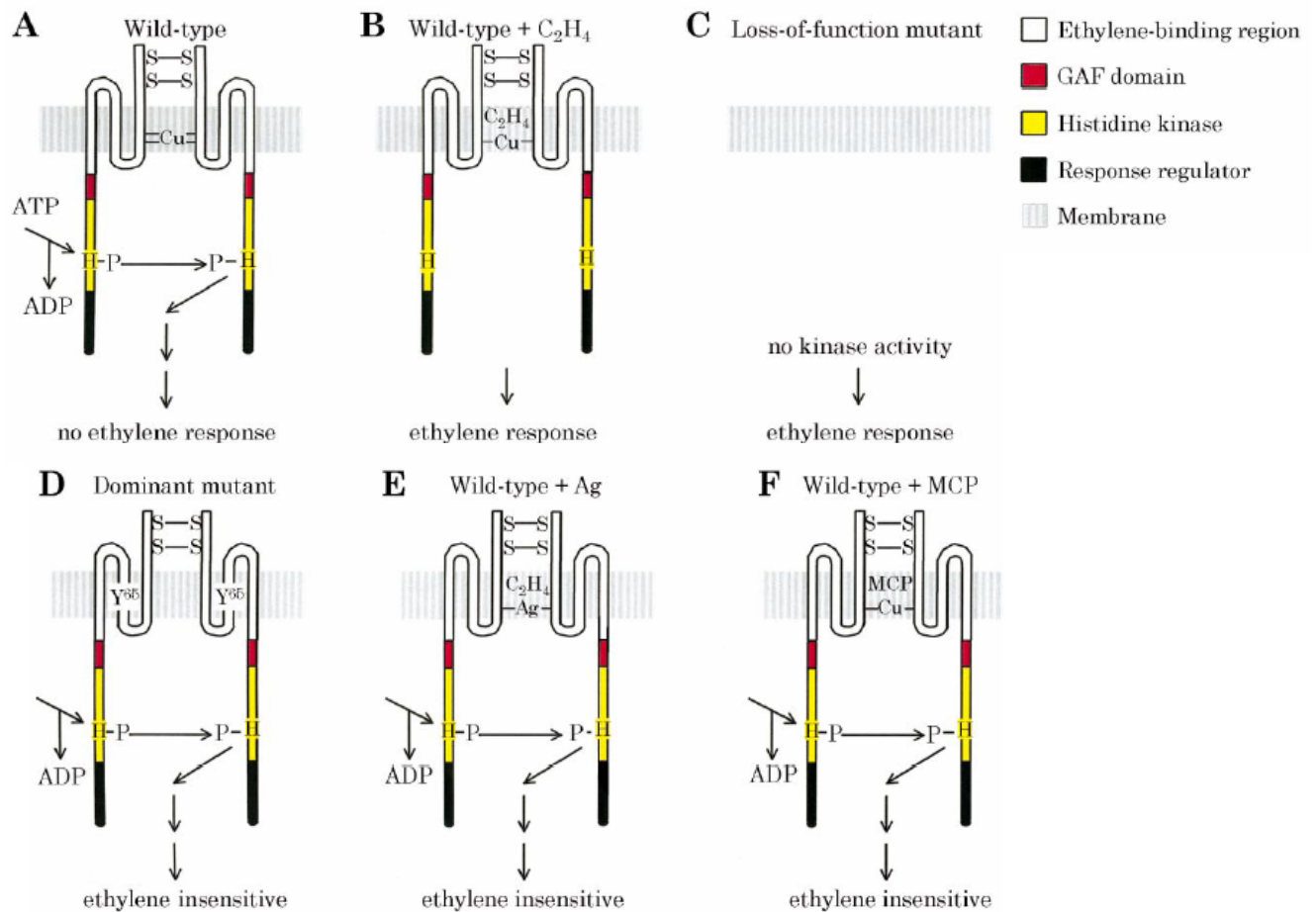
Fig. 10. A model for the role of kinase activity in ethylene signalling. A, The *Arabidopsis ETR1* receptor. B, Ethylene binding to wild-type receptor. C, Loss-of-function mutations in multiple ethylene receptors. D, The dominant *etr1-1* mutation. E, Silver ion competes with copper for receptor binding sites. F, The ethylene action inhibitor 1-methylcyclopropene (1-MCP) competes with ethylene to bind to the receptor. (Ciardi and Klee, 2001).

3.1.4 Expression regulation of ethylene signal transduction pathway

Genetic analysis indicates that *CTR1*, which encodes a protein consisting of 821 amino acids, acts downstream of the receptor in the ethylene signal pathway and localizes at the ER (Kieber *et al*., 1993; Roman *et al*., 1995; Sakai *et al*., 1998; Guo *et al*., 2003). Because *CTR1* has no obvious trans-membrane domain or membrane motifs, its ER localization probably results from direct interaction with the ER-associated receptor (Guo *et al*., 2003). *CTR1* is a negative regulator of the

ethylene-response pathway because *ctr1* null mutants exhibit constitutive ethylene responses even in the absence of ethylene (Kieber *et al*., 1993). The deduced *CTR1* protein sequence is most similar to the Raf family of protein kinase, which initiates mitogen-activated protein (MAP)-kinase signaling cascades in mammals (Fig. 11) (Kyriakis *et al*., 1992; Pelech and Sanghera, 1992). The similarity of *CTR1* known *MAPKKKs* implies that ethylene signaling may operate through a MAP-kinase cascade. Although many genes with homology to *MAPKKs* and *MAPKs* have been identified in the *Arabidopsis* genome sequence, currently none have been involved with ethylene signaling. Thus, no intermediate components have been identified genetically or biochemically to act between the receptors and the *CTR1* kinase. The two-hybrid yeast assay shows that the amino-terminal domain of *CTR1* can interact with the His kinase domains of *ETR1* and *ERS1* (Clark *et al*., 1998). The function of *CTR1* is dependent on both its carboxy-terminal Ser/Thr kinase activity and the association of its amino-terminal domain with ER-bound ethylene receptors. Interestingly, *CTR1* is also able to interact weakly with carboxy-terminal domain *ETR2* (type-II). Thus, it is hypothesized that all five ethylene receptors are able to interact with *CTR1* via their carboxy-terminal kinase domains. However, type-I receptors (*ETR1* and *ERS1*) have a high affinity for *CTR1*, whereas type-II (at least *ETR2*) possess a low binding affinity for *CTR1* (Cancel and Larsen, 2002). Loss of function in the ethylene receptors or *CTR1* results in the induction of ethylene responses. In air, the ethylene receptors maintain *CTR1* in an "on" state that serves to repress the ethylene responses. Binding of ethylene to the receptors turns *CTR1* "off" and leads to derepression of the pathway. Specifically, inactivation of *CTR1* would result in the activation of *EIN2*, and consequently activation of the transcriptional cascade that involves the *EIN3/EIL* and the *ERFs* (Ecker, 1995).
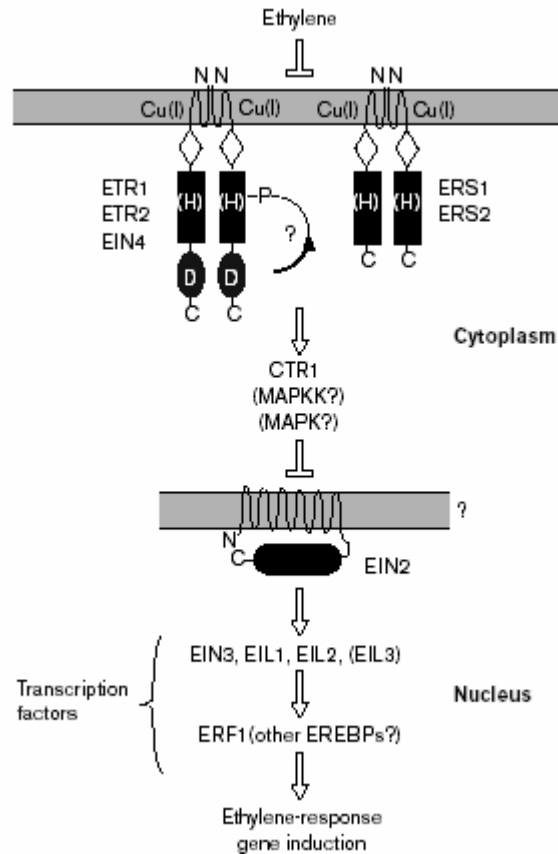
Fig. 11. A current view of the ethylene signal transduction pathway formulated on the basis of cloned *Arabidopsis* genes (Chang and Shockey, 1999).

In *Arabidopsis*, signal propagated from *CTR1* to the nucleus requires *EIN2* to encode an integral membrane protein of 1294 amino acids. Null mutations in *EIN2* result in the complete loss of ethylene responsiveness throughout plant development, suggesting that *EIN2* is an essential positive regulator in the ethylene signal pathway (Fig. 11). *EIN2* comprises 12 predicted transmembrane domains in the third of the polypeptide, a region that resembles members of the *NRAMP* family, including metal-ion transporters. The carboxy-terminal portion of *EIN2* is a novel sequence predicted to be soluble and cytosolic. *EIN2* is also membrane-associated, but lacks detectable metal transport activity. The amino-terminal domain may serve as a sensor of divalent cations or as a membrane anchor. Overexpression of hydrophobic carboxy-terminal domain in an *EIN2* null background results in constitutive activation of some, but not all, of the ethylene response pathway. These results suggest that ethylene regulation of this activity requires the *EIN2* amino-terminal domain. It is hypothesized that the amino-terminal region of *EIN2*

represents an input domain, interacting with upstream signaling factors, while the carboxy-terminal region represents an output domain, interacting with downstream signaling factors (Alonso *et al.*, 1999).

Based on genetic evidence, *EIN3* is a novel nuclear-localized DNA binding protein that encodes a protein of 628 amino acids and acts downstream of *EIN2*. *EIN3,* which contains acidic, praline-rich and glutamine-rich domains found in transcriptional activation domains, is nuclear-localized. Loss of function of *EIN3* results in an ethylene insensitive phenotype, indicating that *EIN3* is a positive regulator of ethylene signal transduction (Fig. 11). In *Arabidopsis*, *EIN3* is a multigene family, in which *EIN3* and *EIN3-like* (*EIL1*) are the most closely related proteins. Loss of function mutation in the *EIL1* gene shows incomplete ethylene insensitivity like *ein3* mutants, but *eil1* mutants are more sensitive to ethylene than *ein3* mutants. In addition, overexpression of *EIN3* and *EIL1* genes confers a constitutive ethylene response phenotype on dark-grown seedlings as well as those grown in the light. Moreover, *EIN3* and *EIL* proteins comprise a novel family of sequence-specific DNA-binding proteins and define a primary ethylene response element (*PERE*) present in the promoters of several ethylene-regulated genes (Chao *et al.*, 1997). *EIN3* is both necessary and sufficient for the activation of ethylene responsive target genes, particularly for *ERF1* (Solano *et al.*, 1998).

ERF1, which is induced by the *EIN3/EIL1,* is itself a transcription factor, indicating that ethylene signal transduction involves a transcription cascade (Fig. 11) (Solano *et al.*, 1998), and is formerly known as *EREBP* (ethylene-responsive element-binding protein). This gene belongs to a family of ethylene-responsive element-binding factor (*ERF*) proteins (Ohme-Takagi and Sinshi, 1995; Fujimoto *et al.*, 2000). *ERF1*, known to be a trans-acting factor responsible for ethylene-regulated expression of genes, induces ripening and senescence and interacts specifically with sequences consisting of AGCCGCC motifs (GCC box) that are found in the promoters of several pathogenesis genes, such those encoding β-1, 3-glucanase, basic chitinase, and defensin (*PDF1.2*) (Ohme-Takagi and Sinshi, 1995; Hao *et al.*, 1998; Solano *et al.*, 1998). Some *ERFs* contain GCC boxes, a cis-acting promoter element, which is both necessary and sufficient to confer ethylene responsiveness in their promoters indicating that they are targets for other members of the *ERF* family, and suggesting the existence of a tertiary level of transcriptional regulation in ethylene signal transduction (Solano *et al.*, 1998). Moreover, *ERFs* share a well-conserved 58-59 amino

acid domain, *ERF* domain, which has a novel structure containing three β-sheets and an α-helix binding to DNA as a monomer, with high affinity (Allen *et al*., 1998; Hao *et al*., 1998). This domain is related to the *AP2* domain in the *Arabidopsis* gene *APATALA2* (Jokofu *et al*., 1994), although ERF proteins contain a single DNA-binding domain whereas the *APATALA2*-type proteins typically contain two (Riechmann and Meyerowitz, 1998). Overexpression of *ERF1* induces a partial triple-response phenotype in dark-grown seedlings, with the seedlings lacking the exaggerated apical hook normally associated with the triple response (Solano *et al*., 1998). This result indicates that the roles of *ERF1* are not confined to the pathogenesis response and other factors. *ERF1* is required for initiating a full ethylene response. Analysis of the *ERF* family in *Arabidopsis* reveals that some members, such as *ERF1*, function as transcriptional activators while other members function as repressors (Fujimoto *et al*., 2000).

ERFs can be divided into three classes based on their amino acid sequences. All classes consist of the *ERF* domain, but the acidic domain in class I has been found in the N-terminal region. In addition, members of this group possess a region rich in basic amino acids (P/L-K-K/R-R-R) that could serve as a putative nuclear localization signal (Raikhel, 1992). In class II, *ERF* domain is located close to the N-terminus of the protein. It comprises 58 amino acids, with one residue shorter than that of class I *ERFs*. Class III has the longest coding region among members of *ERF* protein. Acidity domains are located in both the N- and C-terminal region flanking the *ERF* domain. Additionally, a putative mitogen-activated protein (*MAP*) kinase phosphorylation site is conserved in the C-terminal region. Class I and III are activators, whereas class II has active repressors that can suppress the activity of other transcriptional activators without competing for DNA binding sites (Fujimoto *et al*., 2000; Ohta *et al*., 2001). Class IV is the new *ERF* class that displays a novel and highly conservative N-terminal motif (*MCGGAII/L*). Though the function of *MCGGAII/L* motif has not been established, deletion studies indicate that it is required neither for nucleus localization nor for binding to the GCC box (Tournier *et al*., 2003). In *Arabidopsis*, *AtERF1* and *AtERF2* belong to class I, *AtERF3* and *AtERF4* possess class II and *AtERF5* is the only identified member of class III (Fujimoto *et al*., 2000). ERF proteins play important roles in plant responses to signals that are induced by extracellular stress. They are involved in the induction of gene expression by stress factors, such as pathogens and cold, and by components of stress signal transduction pathways, such as ethylene, abscisic acid and jasmonic acid (Ohme-Takagi and Shinshi, 1995; Büttner and Singh, 1997; Stockinger *et al*., 1997; Zhou *et al*., 1997;

Finkelstein *et al*., 1998; Solano *et al*., 1998; Menke *et al*., 1999). Most of the members of this family have been characterized and shown to participate in stress and/or hormonal responses (Solano *et al*., 1998; Fujimoto *et al*., 2000). The ERF domain-containing *Pti4/5/6* gene products bind to the pathogenesis-related Pto protein kinase in tomato (Zhou *et al*., 1997) and over expression of *Pti4* in *Arabidopsis* induces the expression of GCC box-containing genes (Wu *et al*., 2002), thereby enhancing resistance to pathogen attack (Gu *et al*., 2002).

In *Rosa hybrida*, the expression of *RhCTR1* (66% amino acid identity to *Arabidopsis CTR*) and *RhCTR2* (87% amino acid identity to *AtEDR1* and 90% identity to *LeCTR2*) during flower senescence and in response to ethylene has been examined. *RhCTR1* levels have been upregulated in senescing flowers, while *RhCTR2* is constant during flower senescence. The expression of both genes increases in response to ethylene, indicating the role of these genes in ethylene sensitivity and postharvest performance (Müller *et al*., 2002a). However, it can be assumed that flowers become more sensitive to ethylene during senescence. *CTR* expression will decrease in the developmental stage of flowers and in response to exogenous ethylene as the prediction of the negative regulator model.

RhEIN3, a cDNA encoding part of an *EIN3* transcription factor homologue, has been isolated in *Rosa hybrida*. The deduced protein has 83% and 88% identity to the corresponding regions of *Arabidopsis EIN3* and *EIL1*, respectively. The expression of *RhEIN3* transcript during flower development has been investigated in the miniature rose cultivars 'Bronze', of short flower life and high ethylene production, and 'Vanilla', long-lasting and moderate ethylene production, at three stages of flower development: the bud, the opening flower and the incipient senescence stage. For the opening flower stage, changes in expression of *RhEIN3* after ethylene and ABA treatment have been investigated. The gene *RhEIN3* is constitutively and stably expressed during flower development in both cultivars and in response to ethylene and ABA. The constitutive pattern of *RhEIN3* expression could not be correlated with the previous observed cultivar differences in flower life or ethylene sensitivity in flower or leaves of miniature roses (Müller *et al*., 2002b). These observations are in accordance with previous studies in *Arabidopsis* (Chao *et al*., 1997) and tobacco (Kosugi and Ohasi, 2000), in that ethylene does not affect the level of *EIN3* mRNA, and suggests that control of ethylene sensitivity occurs upstream of *EIN3* and its homologues.

Therefore, the biochemical investigation of inhibitors of ethylene responses, and studies of the ethylene signal transduction pathway using a molecular genetic approach can determine the manner in which ethylene influences these processes. From the previous study of physiological investigation, it is assumed that the difference in the display quality of miniature potted roses after pretreatment with air, 1-OCP, 1-DCP, 1-MCP and afterwards continuous exposure to ethylene, might have an influence on the expression of genes for ethylene biosynthesis enzymes, ethylene perception and the signal transduction pathway. Two selected cultivars, 'Vanilla' (long-lasting flower) and 'Lavender' (short flower life) were selected in order to understand the mechanisms that regulate ethylene induced flower senescence and genetic control of the ethylene response pathway. This could lead to a possibility of selective block processes in flower development that are economically detrimental, and to improve display quality using new chemicals for ethylene receptor inhibition. However, there is no report about the ERF domain-containing genes in miniature roses so far. In order to identify and characterize differentially-expressed genes that might provide more insight into explaining the genetic basis of the ethylene response pathway, the cloning and initial characterization of a novel *ERF* gene, *RhERF1,* from *Rosa hybrida* was reported.

3.2 Materials and Methods

3.2.1 Plant material

The plants were grown as previously described in chapter 2.2.6. They were harvested after continuous exposure to exogenous ethylene and ethylene-free air for 9 days and then put in 15 ml polyethylene tubes (Sarstedt, Germany) and immediately frozen in liquid nitrogen. They were then ground in liquid nitrogen and stored at -80°C in a deep freezer until extraction of DNA or RNA.

3.2.2 Database analyses and primer design

A sequence comparison between known ERF sequences of different plant species was done according to Mibus (2003). The ERF primer pairs were designed using program Primer 3 (Steve and Skaletsky, 2000) (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and was commercially synthesized by MWG Biotech AG, as shown in Table 1.

Table1. Primer pair for amplification of the Ap2 (DNA binding Domain) coding sequence of *ERF1*

Name	Sense Primer	Name	Antisense Primer	Annealing Temp (^{o}C)	Size (bp)
*ERF1*s	5′TACAGAGGCGTTAGGAAGCG3′	*ERF1*as	5′CGAAAGTGCCAAGCCAGA3′	55	92

3.2.3 DNA isolation and PCR

Genomic DNA was isolated from 80 mg of ground petals of miniature roses 'Vanilla' and 'Lavender' using DNeasy^{R} Plant Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The concentration of 'Vanilla' and 'Lavender' genomic DNA was determined by comparing it with standard concentrations (5, 10, 25, 50, 100 and 200 µg ml^{-1}) of λDNA (Fermentas GmbH, St, Leon-Rot, Germany) in 1% agarose flatted gel electrophoresis visualised by staining with 40 µg ethidium bromide. A temperature gradient PCR was performed to optimize the annealing temperatures for the ERF primer (Table 1) and various gene-specific primers (table 2) to minimize the number of incorrect base pairings (mismatches). This

phenomenon is enhanced by low annealing temperature which is responsible for mismatching (Rychlick *et al.*, 1990). For PCR analysis, 15 ng genomic DNA was used as a template in a 20 µl volume reaction that contained 0.03 µM of *ERF1* primer pair: *ERFs* and *ERFas* (Table 1) or 1.2 µM gene-specific primer pairs (table 2), 150 µM of each dNTP and 0.2 units of DyNAzyme^{TM} II DNA polymerase (Finnzymes Oy, Espoo, Finland) in the 10x (100 mM Tris-HCL, pH 8.3, 500 mM KCl, 20 mM MgCl$_2$ and 0.01% gelatin) Williams buffer. The reaction mixture was incubated in a Thermocycler (Biometra, Göttingen, Germany) and the PCR conditions were: 2 min at 94°C (initial denaturation), followed by 40 cycles lasting 1 min at 94°C (denaturation), 1 min at 49°C to 56°C (40 cycles) (annealing), and 2 min at 72°C (extension) and a cooling step at 4°C.

3.2.4 Cloning, DNA sequencing and sequence analysis

Orange G (30% Glycerin, 20 mM EDTA-pH 8, 1.25% Orange G and 10 ml deionised water) loading buffer was added to the PCR products and centrifuged for 5 seconds. This mixture was then loaded into 1% agarose gels and separated in a flatted gel electrophoresis using 1xTAE (40 mM Tris-Acetate, 1mM EDTA, pH 8.0) Buffer. The gels had a capacity of either 50 ml or 150 ml and were run at 120V or 80V, respectively. They were also visualised by staining with 0.3 µg/ml ethidium bromide and observed using a BioDocAnalyze UV trans-illuminator (Biometra, Göttingen, Germany). The sizes of amplicons were estimated by comparing them to a 100 base pair-ladder DNA marker. The desirable fragments were cloned by ligation into a TA plasmid cloning vector using pCR^{R}4-TOPO^R TA Kit (Invitrogen, Carlsbad, CA; Appendix. 5) and transformed into *Escherichia coli*.

The plasmid vector (pCR^{R}4-TOPO^R) was supplied linearised, also called "activated vector". The *Vaccinia* virus Topoisomerase I binds to duplex DNA at specific sites and cleaves phosphodiester backbone after 5'-(T/C)CCTT in one strand (Shuman, 1991) and the energy released is conserved through formation of a covalent bond between 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phosphate-tyrosyl bond between DNA and enzyme can subsequently be attacked only by a 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). The plasmid vector has two adjacent cutting surfaces and since both DNA fragments are blocked by Topoisomerase, this prevents self ligation. Moreover, the vector allows direct selection of positive recombinants via disruption of

the lethal *Escherichia coli*, *ccd*B gene (Bernard *et al*., 1994) since the cells that contain a non-recombinant vector are killed upon plating, which eliminates the need for blue/white screening.

After cloning (50 ng of the PCR product) and transformation into *Escherichia coli* according to the manufacturer's instructions (TOPO TA Cloning kit; Invitrogen), 125 µl S.O.C. medium (2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM glucose) was added to the plasmid–*E. coli* mixture and shaken horizontally using a shaker at 240 rpm for 1.5 hours at 37°C in order for bacteria to multiply. The transformants were then cultured in LB (1% Tryptone, 0.5% Yeast extract, 1% NaCl, 950 ml deionised water, pH 7.0) medium containing 150 µg µl^{-1} Ampicillin at 37°C. Plasmid DNA from transformed *E. coli* was recovered using the NucleoSpin^R Plasmid Kit (Macherey-Nagel GmbH, Düren, Germany). Positive transformants were directly analysed using 0.2 µg T3 and 0.2 µg T7 primer pairs in a PCR reaction. The nucleotide sequences for T3 5'-ATTAACCCTCACTAAAGGGA-3' and T7 5'-TAATACGACTCAACTATAGGG-3'. Sequencing by dideoxynucleotide method was performed commercially by MWG Biotech AG (Ebersberg-Munich, Germany). The isolated sequences were analysed using CLUSTAL W program, European Bioinformatics Institute (EMBL; Higgens, 1994), and the homology search was done using the BLUSTN programme, National Center for Biotechnology Information (NCBI; Altschul *et al*., 1997).

3.2.5 RNA Isolation and RT-PCR

Total RNA was isolated from 30 mg of ground petals and leaves of miniature rose cultivars 'Vanilla' and 'Lavender' after treatment with continuous ethylene for 9 days or ethylene-free air as described in section 2.2.6, using Invisorb^R Spin Plant RNA Mini Kit (Invitek GmbH, Berlin, Germany) following the protocols by the manufacture. Total RNA was determined by use of a spectrophotometer (Biorad, California). To check for the presence of contaminating genomic DNA, total RNA was compared with standard concentrations (5, 10, 25, 100 and 200 µg ml^{-1}) of λDNA (Fermentas GmbH, St. Leon-Rot, Germany) in a flatted gel electrophoresis visualized by staining with 0.3 µg/ml ethidium bromide then stored at -20°C for short-term or -80°C deep freezer for long-term storage, until further use. Under these conditions, no degradation of RNA is detectable for at least 1 year. RNA was handled using latex hand gloves and was kept under ice when taking aliquots. Additionally, the laboratory working

surfaces and equipments were cleaned with RNase degrading chemicals (RNase Exitus; AppliChem).

cDNA was synthesized and used as a template for PCR. Before RT-PCR, the residual genomic DNA in the total RNA preparation was removed through a RNase-free DNase treatment as follows: 0.5 µg RNA was treated with 1 µl 10x buffer, 0.5 units Deoxyribonuclease I (Rnase-free) (Fermentas GmbH, Heidelberg, Germany), 0.5 units RiboLock^{TM} Ribonuclease Inhibitor (Fermentas GmbH, Heidelberg, Germany), then the mixture was incubated in a Thermocycler (Biometra, Göttingen, Germany) for 30 min at 37°C. After incubation, 40 µM EDTA was added to the mixture to deactivate DNase. This was done at 65°C for 10 min. 0.5 µg of total RNA was used as a template for cDNA synthesis using M-MLV RT, RNase H Minus: a point Mutant (Promega Corporation, Manheim, Germany). First strand cDNA synthesis was carried out using first strand oligo(dT)$_{23}$ for 10 min at 40°C, then 2 h at 50° C and a final deactivation for 10 min at 70°C. The resulting cDNA was diluted to a concentration of 20 pg µl^{-1} for the PCR reaction. The PCR reaction mixture contained 2 µl of cDNA, 2 µl of 10x Williams Buffer (see section 3.2.3) recommended by the supplier (Biometra, Göttingen, Germany), 150 µM dNTPs, 1.2 µM gene-specific primer pairs and 0.5 units Taq DNA polymerase (Invitek GmbH, Berlin, Germany) in a total volume of 20 µl. Primers (0.25 µM of each) used for the amplification of *β-Actin* (used as a reference) were: ACC synthase; *RhACS1, RhACS2, RhACS3, RhACS4, RhACS5, ETR* genes; *RhETR1, RhETR2, RhETR3, RhETR*4, *CTR* genes; *RhCTR1, RhCTR2, EIN3* and *EIN-like* genes; *RhEIN3, RhEIL* and *ERF* gene; *RhERF1* were designed from sequences available in the databases (*Rosa hybrida*) (http://www.ncbi.nlm.nih.gov) (Table 2). For the PCR reaction, between 35-45 cycles, were observed with the clear differences of amplification detected at 40 cycles. The reaction mixture was incubated in a Thermocycler (Biometra, Göttingen, Germany). PCR conditions were: 5 min at 94°C (initial denaturation), followed by 40 cycles consisting of 30 s at 94°C (denaturation), 30 s at 49°C to 55°C (annealing) (see Table.2), and 30 s at 72°C (extension) and a cooling step at 4°C. The products of PCR were separated as described for the normal PCR above. PCR and the experiments themselves were repeated three times for each sample. A representative result is presented in chapter 3.3.2.

Table 2. Gene specific primer pairs for *β-Actin* (Accession No. X55751), *ACC* synthase; *RhACS1* (Accession No. AY061946), *RhACS2* (Accession No. AY525066), *RhACS3* (Accession No. AY525067)*, RhACS4* (Accession No. AY525068), *RhACS5* (Accession No. AY525069), *ETR* genes; *RhETR1* (Accession No. AF394914)*, RhETR2* (Accession No. AF127220)*, RhETR3* (Accession No. AF154119)*, RhETR4* (Accession No. AF159172), *CTR* genes; *RhCTR1* (Accession No. AY032953)*, RhCTR2* (Accession No. AY029067), *EIN3* and *EIN-like* genes; *RhEIN3* (Accession No. AF443783)*, RhEIL* (Accession No. AY052825) and *ERF* gene; *RhERF1* designed using the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Name	Sense Primer	Name	Antisense Primer	Annealing Temp (^{o}C)	Size (bp)
Actin_for	**5′AGATCTTTATGGAAACATTGTGCTC3′**	*Actin_Rev*	**5′ATCCAGACACTGTATTTCCTCTCT3′**	49	150
RhACS1s	5′AAAACCCAGAAGCCTCCATT3′	*RhACS1as*	5′ATTTCTGGTTCCCGTTCCTT3′	55	205
RhACS2s	5′AAAACCCAGAAGCCTCCATT3′	*RhACS2as*	5′ATTTCTGGTTCCCGTTCCTT3′	55	249
RhACS3s	5′CCATGGCCTTTTGTCCTTTA3′	*RhACS3as*	5′ACTGCAGCCAATGAGCTTTT3′	55	126
RhACS4s	5′GCTTCCAACTTGGGATCAAA3′	*RhACS4as*	5′TTCCAACCCCATACTACCCA3′	55	237
RhACS5s	5′CAGCCGGATTCAAGAGAAAC3′	*RhACS5as*	5′CTCTTATGTTTTGCCTCGCC3′	55	203
RhETR1s	5′TGGTATGAACCTTCAACTTTCTCAT3′	*RhETR1as*	5′TGCTATTCTTGAAGAGTCTATGCGA3′	55	393
RhETR2s	5′CTCAAACTTCCAAATCAATGACTG3′	*RhETR2as*	5′GGATCTGCTAATGGAGCAGAATAT3′	55	213
RhETR3s	5′CACTGCTATAACGCTCATCACTCT3′	*RhETR3as*	5′ATCCTTGAAGAGTCCCAACTAATG3′	55	661
RhETR4s	5′TTTGAATCTGCAACTTTCTCACAC3′	*RhETR4as*	5′TGTCATGAACCACGAAATGC3′	55	500
RhCTR1s	5′GCTTCTGATGTTGCTGTGAA3′	*RhCTR1as*	5′TCAATGGCCTCAAAGATTCC3′	52	706
RhCTR2s	5′TTCCTTCCAAGGGGAAGTCT3′	*RhCTR2as*	5′CCCACTCCAAGCCAATTTTA3′	55	357
RhEIN3s	5′CCCTGCAGCCATAGACAAGT3′	*RhEIN3as*	5′ACCCTGATTTCATCCACCAA3′	56	236
RhEILs	5′TCCCTGGTTTGATGGAAGAC3′	*RhEILas*	5′GAGGCCACCATTCCTCATTA3′	56	192
RhERF1s	5′TTCTTCCACGGGTCTCTGATCTC3′	*RhERF1as*	5′GCTTCCTAACGCCTCTGTAA3′	55	64

The *β-Actin* primer used as a reference gene is in boldface.

3.2.6 Northern blot hybridization

Polysomal RNA was extracted as described in section 3.2.5. Northern blotting was performed according to Terefe (2005). Equal amounts (8 µg per sample) of total RNA were denatured in freshly prepared loading buffer (2.2 M formaldehyde, 50% foramide, 0.5x MOPS and 0.05% bromophenol blue (0.25% (w/v) bromophenol blue, 30% (v/v) Glycerin) in a final volume of 30 µl for 15 min at 65°C. The denatured RNA was then fractioned by electrophoresis in 1% agarose gel containing 0.7 M formaldehyde in 1x MOPS-buffer (200 mM 3-(N-Morpholino)-

propanesulfonic acid (MOPS), 80 mM Sodium acetate 3-hydrate, 12.5 mM EDTA, pH 7.0) for 4-5 h (at the constant voltage of 5V/cm gel length). The gels were stained with 1,5 µg/ml ethidium bromide to check the intactness of the RNA under UV light, then washed first in water for 15 min and twice in 10x SSC (1.5 M NaCl, 0.15 M Na-citrate, pH 7.0) for 15 min each. RNA was then transferred to positively-charged nylon membranes (Hybond-N$^+$, Amersham Biosciences UK Ltd, Buckinghamshire, England) in 10x SSC using a vaccuum-system (Pharmacia; 60 mbar, 90 min). After crosslinking by irradiation with UV light, the membranes were rinsed with water and stored until hybridization.

3.2.7. DIG labeling of the probes for northern blot hybridization

The ethylene receptor and signal transduction pathway, *RhETR1-2*, *RhEIN3* and *RhEIL* were labeled with DIG (digoxigenin) (Roche Diagnostics GmbH, Mannheim, Germany) for non-radioactive hybridization. DIG labeling was performed by PCR using 10 pg plasmid, containing each gene of interest: 2.5 µM of each standard T$_3$ 5'-ATTAACCCTCACTAAAGGGA-3' and T$_7$ 5'-TAATACGACTCAACTATAGGG-3' primers, 10x reaction William Buffer (GeneCraft GmbH, Lüdinghausen, Germany) as recommended by the supplier (Biometra, Göttingen, Germany), 10x DIG DNA labeling mix and 1 unit DyNAzyme^{TM} II DNA polymerase (Finnzymes Oy, Espoo, Finland) filled to a final volume of 20 µl with ddH$_2$O. The reaction mixture was incubated in a Thermocycler (Biometra, Göttingen, Germany). PCR conditions were: 2 min at 94°C (initial denaturation), followed by 40 cycles lasting 30 s at 94°C (denaturation), 1 min at 50°C (annealing), 2 min at 72°C (extension) and 5 min at 72°C (final extension). As a control, additional PCR reactions were set for every probe, labeled by using normal dNTPs without DIG. 5 µl DIG-labeled PCR product and the control were checked by flatted electrophoresis in 1% agarose gel visualised by staining with 40 µg ethidium bromide. The size of fragments was estimated using 100 base pair-ladder DNA marker. Due to the presence of DIG, the DIG-labeled probes migrate slower and appear to be larger than PCR fragments obtained using the normal dNTPs. The DIG-labeled probes were denatured at 95°C for 10 min and immediately chilled on ice for denaturation. The denatured probe was then used for northern blot hybridization.

3.2.8. Hybridization and washing conditions for northern blots

The membranes were prehybridized in prehybridization solution containing Dig Easy Hyb (Roche Diagnostics GmbH, Mannheim, Germany) at 50°C for 30 min. Hybridization was carried out overnight at the same condition with 100 ng of the denatured DIG-labeled probe in 7.5 ml Dig Easy Hyb. After hybridization, membranes were washed twice for 5 min each in 2x SSC/0.1% SDS at 50°C. The membranes were then agitated in 1x blocking solution (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min to eliminate nonspecific background and for another 30 min in an Anti-Dig-AP solution (Roche Diagnostics GmbH, Mannheim, Germany) (2.5 µl (0.75 unit/µl) Anti-Dif-AP in 50 ml 1x blocking solution). Detection of the DIG-labeled probes was performed according to the instructions of the supplier, using CDP-star (Roche Diagnostics GmbH, Mannheim, Germany) as substrate. X-ray films (Hyperfilm, Amersham Biosciences UK Ltd, Buckinghamshire, England) were exposed to the membranes for about 1 h. The transcript sizes were estimated by comparison with the RNA Molecular Weight Marker I (Roche Diagnostics GmbH, Mannheim, Germany) covering the range 0.3-6.9 kb. Blots were used for multiple hybridizations after stripping in 0.1x SDS boiling.

3.3 Results

3.3.1 Cloning and sequence analysis of *RhERF1*

Using PCR with primer pairs deduced from the conserved region (Ap2 Domain) of *Arabidopsis ERF* homologues, a fragment encoding a partial *ERF* homologue was amplified from genomic DNA from 'Vanilla' and 'Lavender'. A database search revealed that the deduced amino acid sequence of *RhERF1* contained a highly conserved DNA binding domain. This consisted of 30 amino acids (92 bp for partial-length of DNA sequence) and a shared high identity with other reported ERF domain-containing proteins (Fig. 12).


```
          β-sheet-1 β-turn-1 β-sheet-2 β-turn-2 β-sheet-3

                 *   *    *     *   *        *  *                          Position
RhERF1   :  YRGVRKRPWGRYAAEIRDPWKK-TRVWLGTF------------------------------   1-30
AtERF1   :  YRGVRQRPWGKFAAEIRDPAKNGARVWLGTFETAEDAALAYDRAAFRMRGSRALLNFPLR   147-201
AtERF2   :  YRGVRQRPWGKFAAEIRDPAKNGARVWLGTFETAEDAALAYDIAAFRMRGSRALLNFPLR   116-176
NtERF1   :  YRGVRRRPWGEFAAEIRDPAKNGARVWHRTYETDEEAAIAYDKAAYRMRGSKAHLNFPHR   71-131
LeERF1   :  YRGVRQRPWGKFAAEIRDPAKNGARVWLGTYESAEEAALAYGKAAFRMRGTKALLNFPHR   2-62
GmERF1   :  YRGVRRRPWGKFAAEIRDPKKNGARVWLGTYDTEEKAALAYDKAAFKMRGQKAKLNFPHL   76-136

          YRGVR RPWGKFAAEIRDP K G RVWLGTY
```

Fig. 12. Alignment of the DNA-binding domains of *RhERF1* in comparison with other ERF proteins: *AtERF1* (GeneBank Accession No. AAL25588), *AtERF2* (GeneBank Accession No. NM_124093), *NtERF1* (GeneBank Accession No. AF057373), *LeERF1* (GeneBank Accession No. AY192367), *GmERF1* (GeneBank Accession No. AAM45475). The identical amino acids among all the aligned ERF proteins are marked in grey. The amino acids with asterisks in the *ERF* consensus indicate residues that interact with nucleotides with one word in the GCC box. The numbers at right indicate the position of the amino acids in the ERF domain for each protein.

3.3.2 Expression patterns of genes for the ethylene biosynthesis enzyme, ethylene perception and signal transduction pathway after pretreatment with ethylene receptor inhibitors

Ethylene caused leaf, bud and flower drop, but there were differences among cultivars. In both cultivars, ethylene accelerated flower senescence. Continuous exposure to exogenous ethylene at 1.25 nl l^{-1} hastened leaf drop (as expressed in percentage) for 'Vanilla' and 'Lavender' (Fig 13A, B). 'Vanilla' control plants attained 90% leaf drop after 9 days of continuous exposure to ethylene, as opposed to 'Lavender', which attained 100% leaf drop after the same period. Pretreatment with ethylene receptor inhibitors, 1-OCP, 1-DCP and 1-MCP, significantly improved the display quality in both cultivars (Fig 13A, B). 1-MCP was the most effective of all ethylene receptor inhibitors tested in improving the display quality in both cultivars. However, 1-OCP and 1-DCP were not different from each other with respect to leaf drop in both cultivars (see chapter 2) (Fig. 13A, B).
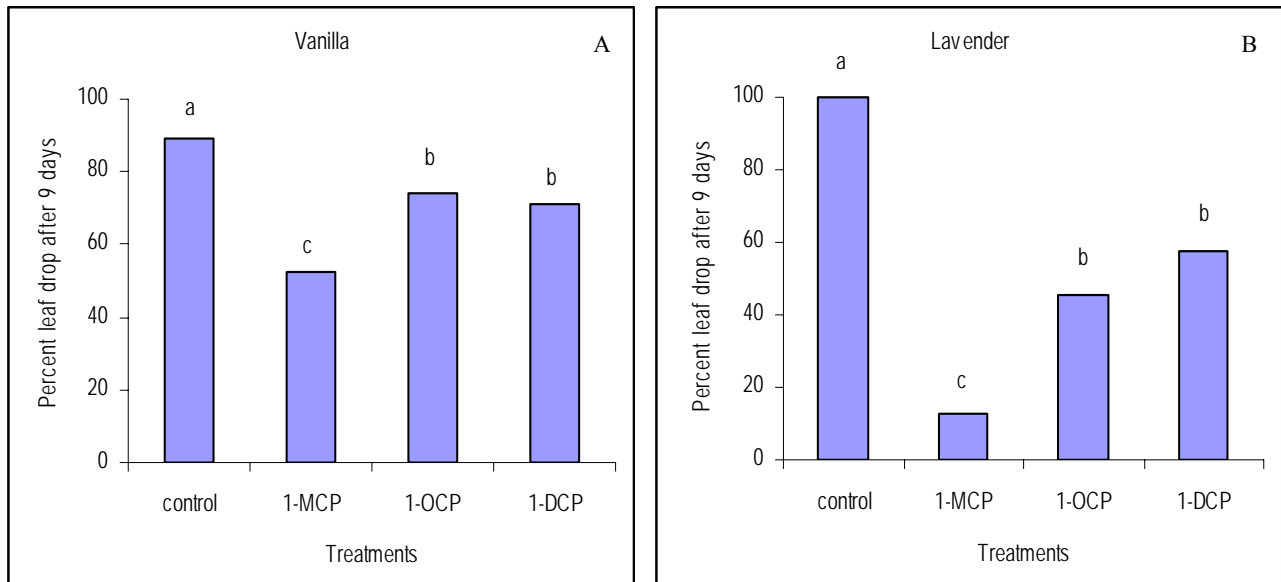
Fig. 13. Percent leaf drop of miniature potted rose cultivars 'Vanilla' (A) and 'Lavender' (B), after day 9 pretreated with 200nl l^{-1} 1-MCP for 6 h, and 1000 nl l^{-1} 1-OCP and 1-DCP for 4 h at 20°C, respectively. After treatments, the plants were exposed to 1.25 (+/- 0.25) µl l^{-1} continuous ethylene throughout the experiments. Bars marked with the same letter are not statistically different at $P<0.05$. Means were separated by LSD (selected data from Fig. 7; p. 28).

To investigate transcript expression, abundance of genes for the ethylene biosynthesis enzyme, ethylene perception and other ethylene-related genes (*RhACS1*, *RhACS2*, *RhACS3*, *RhACS4*, *RhACS*5, RhETR1, RhETR2, RhETR3, RhETR4, RhCTR1, RhCTR2, *RhEIN3, RhEIL* and *RhERF1*) in petal and leaf tissues was examined in the two rose cultivars 'Vanilla' and 'Lavender'. This was done after pretreatment with air, 200 nl l^{-1} 1-MCP for 6 h, 1000 nl l^{-1} 1-OCP and 1-DCP for 4 h at 20°C, followed by either continuous exposure to exogenous ethylene or ethylene-free air for 9 days (Fig. 15-16, A, B). Two *ACS* genes, *RhACS1* and *RhACS2*, were expressed in rose tissues while *RhACS3-5* was undetectable in the investigated tissues (leaves and petals) (method previously described). Northern blotting with *RhETR2* probes from miniature rose was performed to compare the results of RT-PCR. The position was consistent with the expected size of *RhETR2*, but transcription signals of ethylene perception could not be clearly seen on the autoradiogram (Fig. 14). RT-PCR was utilized to compare the expression level of ethylene receptors in miniature rose tissues.
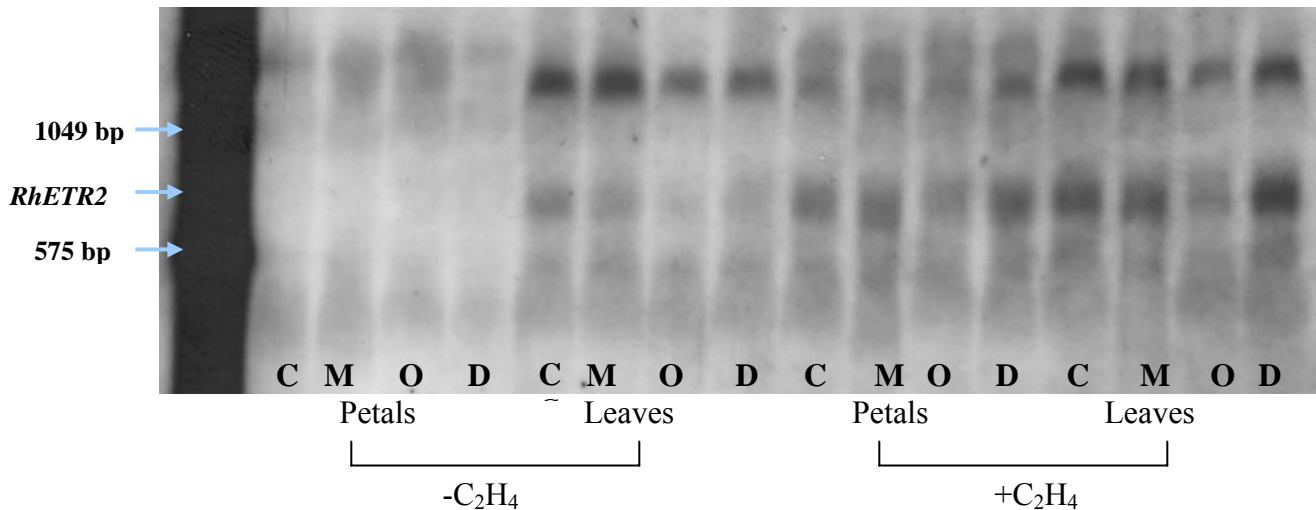
Fig. 14. Northern blot hybridization analysis of ethylene receptor, *RhETR2,* on 8 μg total RNA obtained from petals and leaves of miniature rose cultivar 'Vanilla' in untreated (control) plant (C) and pretreatment with 200 nl l^{-1} 1-MCP (M) for 6 h and 1000 nl l^{-1} 1-OCP (O) and 1-DCP (D) for 4 h at 20°C after 9 days of continuous exposure of exogenous ethylene (+C$_2$H$_4$) and without ethylene treatment (–C$_2$H$_4$).

Pretreating 'Vanilla' petals with 1-MCP, 1-OCP and 1-DCP led to the suppression of the mRNA level of genes for ethylene biosynthesis, ethylene perception and ethylene signal transduction in both the presence and absence of ethylene (Fig. 15A). In the untreated petals (control), ethylene induced the expression of genes for ethylene perceptions (*RhETR1*, *RhETR2*, *RhETR3* and *RhETR4*) and other ethylene-related genes (*RhCTR1*, *RhCTR2*, *RhEIN3*, *RhEIL*, *RhERF1*). However, *RhACS1* and *RhACS2* were not affected by ethylene treatment, and in the absence of ethylene they had no effect on the expression of any of the genes investigated. (Fig. 15A).

The absence of ethylene in 'Vanilla' control leaves led to a strong expression of *RhCTR2*, moderate expression of *RhETR1, RhETR2*, *RhETR4*, *RhEIN3*, *RhEIL*, weak expression of *RhACS1*, *RhETR3*, *RhERF1* and no expression of *RhACS1* (Fig. 15B). Moreover, pretreating 'Vanilla' leaves with 1-MCP, 1-OCP and 1-DCP, in the absence of ethylene, eliminated the abundance of mRNAs for all investigated genes (Fig. 15B). In 'Vanilla' control leaves, the ethylene treatment resulted in strong expression of *RhETR1*, *RhETR2*, *RhETR4*, *RhCTR2* and *RhEIL*, moderate expression of *RhACS2*, *RhCTR1* and *RhEIN3*, weak expression of *RhETR3* and *RhERF1* and no expression of *RhACS1* (Fig. 15B). Furthermore, *RhEIN3* was strongly and constitutively expressed after pretreating 'Vanilla' leaves with 1-MCP, 1-OCP and 1-DCP (Fig. 15B), while *RhEIL* was also expressed in 'Vanilla' leaves in response to 1-OCP and 1-DCP. Pretreating 'Vanilla' leaves with 1-DCP in the presence of ethylene led to a weak accumulation of *RhETR1-2*, *RhCTR1-2* transcripts (Fig. 15B).

Fig. 15. Expression of genes for ethylene biosynthesis, ethylene perception and ethylene signal transduction in miniature potted rose cultivar 'Vanilla' (A, B) in untreated (control) plant and pretreatment with 1-MCP, 1-OCP and 1-DCP after 9 days of continuous exposure of exogenous ethylene (+ C_2H_4) and without ethylene treatment (- C_2H_4). Lane 1, control (C); lane 2, 1-MCP (M); lane 3, 1-OCP (O) and lane 4, 1-DCP (D). PCR reaction contained 20 pg μl^{-1} of cDNA. *β-Actin* was used as an internal control to normalize the amount of cDNA.

In the absence of ethylene, none of the genes were detectable at 40 cycles of PCR reaction in the petals of 'Lavender', irrespective of the treatment applied (Fig. 16A). Exposing 'Lavender' control petals to ethylene resulted in strong expression of *RhETR2*, *RhCTR1*, *RhCTR2*, *RhEIN3* and *RhEIL*, moderate expression of *RhETR1* and *RhETR3*, weak expression of *RhACS1*, *RhACS2* and *RhERF1* and no expression of *RhETR4* (Fig. 16A). However, pretreating 'Lavender' petals with 1-MCP and 1-OCP inhibited the expression of all genes while strong accumulation of *RhETR3*, *RhEIN3* and *RhEIL* transcripts was detectable in 'Lavender' petals pretreated with 1-DCP (Fig. 16A).

In the absence of ethylene, pretreating 'Lavender' leaves with 1-MCP upregulated the expression of *RhACS2*, *RhCTR1* and *RhEIL* as compared to the control leaves (Fig. 16B), while downregulating the expression of *RhETR1*, *RhETR2* and *RhETR3* as opposed to the control. Also, 1-MCP suppressed the expression of *RhACS1*, *RhETR3* and *RhERF1*, whereas it had no effect on the expression of *RhETR4* and *RhCTR2* (Fig. 16B). Similarly, pretreatment of 'Lavender' leaves with 1-OCP upregulated the expression of *RhACS2*, *RhETR4*, *RhCTR1*, *RhEIN3*, but downregulated the expression of *RhETR2*, *RhETR3* as compared to the control leaves (Fig. 16B). 1-OCP inhibited the expression of *RhACS1*, *RhCTR2* and *RhERF1* and had no effect on the expression of *RhETR1* and *RhEIL* as compared to the control. Likewise, pretreatment of 1-DCP upregulated the expression of all *RhACS* and *RhETR* genes, but downregulated the expression of *RhCTR2* as opposed to control leaves. Moreover, 1-DCP inhibited the expression of *RhEIN3*, *RhEIL* and had no effect on the expression of *RhCTR1* and *RhERF1* as compared to the control (Fig. 16B).

In the presence of ethylene, pretreating 'Lavender' leaves with 1-MCP suppressed the expression of *RhACS1*, *RhETR4*, *RhCTR-2*, *RhEIL* and *RhERF1*, while it downregulated the expression of *RhETR1-2* and *RhEIL*, and had no effect on the expression of *RhACS2*, *RhETR3* when compared to control 'Lavender' leaves. Similarly, pretreatment of 1-OCP inhibited the expression of *RhACS1*, *RhETR2*, *RhETR4*, *RhCTR1-2*, *RhEIN3* and *RhERF1* but upregulated the expression *RhEIL*. 1-OCP downregulated the expression of *RhETR1* whereas it did not affect the expression of *RhACS2*, *RhETR3* when compared to the control (Fig 16B). Pretreating 'Lavender' leaves with 1-DCP suppressed the expression of *RhACS1* and *RhETR4*. However, 1-DCP upregulated *RhETR3* and *RhCTR2* while it downregulated the expression of *RhETR2*, *RhEIN3*, *RhEIL* and *RhERF1*, but had no effect on the expression of *RhACS2*, *RhETR1* and *RhETR3* when compared to the control (Fig. 16B).
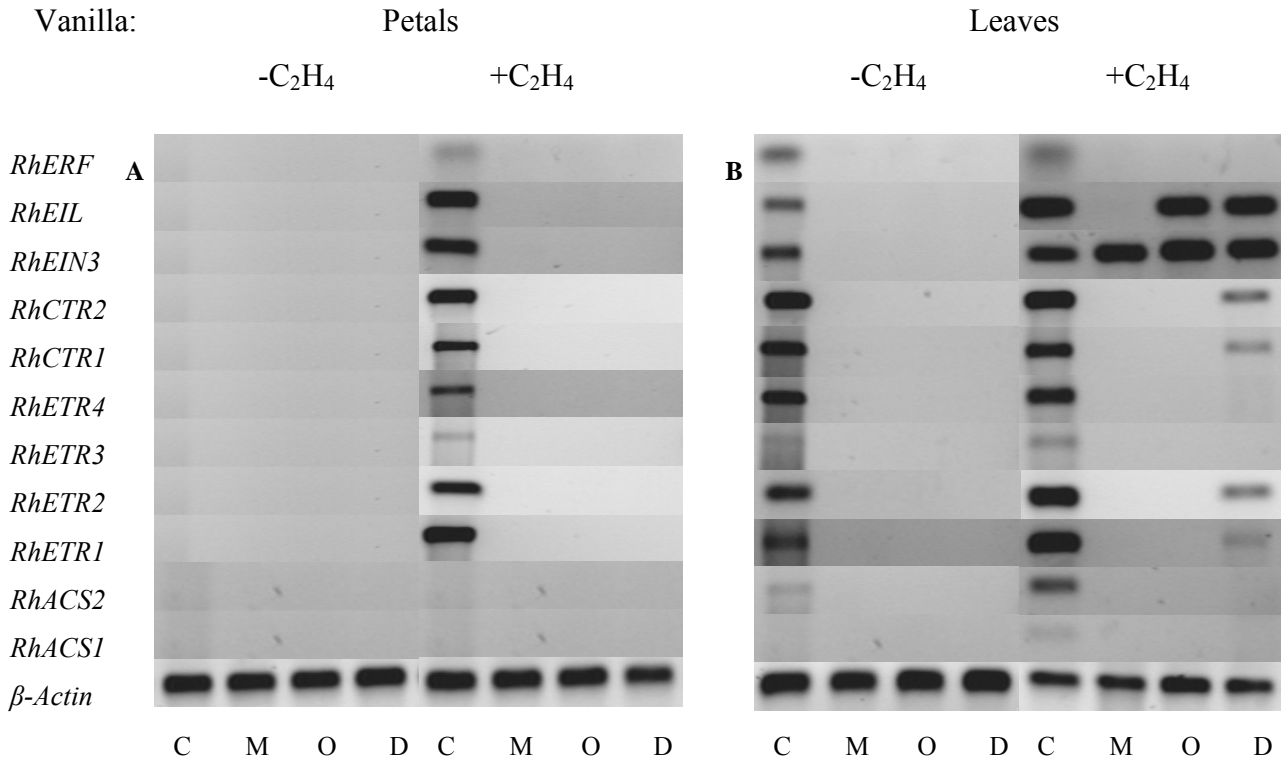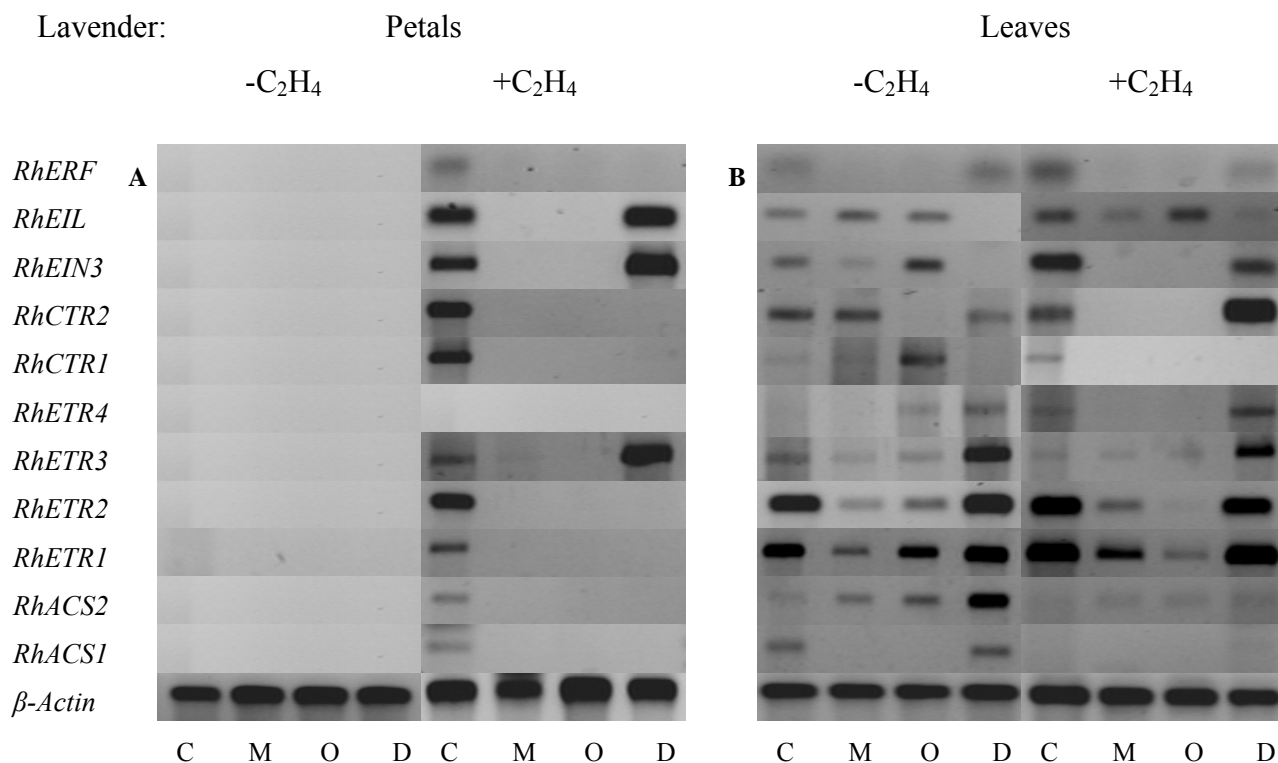
Fig. 16. Expression of genes for ethylene biosynthesis enzyme, ethylene perception and ethylene signal transduction in miniature potted rose cultivar 'Lavender' (A, B) in untreated (control) plant and pretreatment with 1-MCP, 1-OCP and 1-DCP after 9 days of continuous exposure of exogenous ethylene (+ C_2H_4) and without ethylene treatment (- C_2H_4). Lane 1, control (C); lane 2, 1-MCP (M); lane 3, 1-OCP (O) and lane 4, 1-DCP (D). PCR reaction contained 20 pg µl^{-1} of cDNA. β-Actin was used as an internal control to normalize the amount of cDNA loaded.

3.4 Discussion

Differences in flower display quality are partly due to differences in endogenous ethylene production and/or sensitivity to exogenous ethylene (Müller *et al*., 1998, 2001). 'Vanilla' and 'Lavender' showed differences in ethylene sensitivity for their leaf abscission. After 9 days of continuous exposure to ethylene, 'Vanilla' control plants showed about 80% leaf drop, while display quality of 'Lavender' control plants was clearly reduced by 100% leaf drop (see chapter 2). In rose, 1-MCP protects against exogenous ethylene, increasing display life and reducing abscission of buds, flowers and leaves (Serek *et al*., 1994c). To investigate the role of ethylene response in miniature roses pretreated with ethylene action inhibitors, 1-MCP, 1-OCP and 1-DCP, the differential regulation of genes for ethylene biosynthesis enzyme, ethylene perception and signal transduction pathway in petals and leaves following ethylene treatment and ethylene free-air was assessed by reverse transcription-PCR (RT-PCR) analysis. Northern blot hybridization analysis was also used to compare the results from RT-PCR, but transcription signals of the ethylene receptor, *RhETR2*, on the gel made it difficult to distinguish the clear bands. It could be due to a cross hybridization between other members of the ethylene receptor family in miniature rose that are highly homologous. Nucleotide sequence for probe of *RhETR2* is 77% identical to *RhETR4* and 63% identical to *RhETR1*, indicating that *RhETR2* is the most homolog with *RhETR4* and *RhETR1*. Northern blotting depends on minimal RNA degradation, efficient transfer out of the gel, and ability to reliably detect the sequence of interest (Sobel *et al*., 2002). This method, however, is time-consuming and requires a large quantity of RNA (Chelly and Kahn, 1994). Northern blot analysis is effective for quantifying gene expression, while RT-PCR converts RNA into first strand cDNA used as a template for PCR. Moreover, RT-PCR is more rapid and sensitive and can be more specific than northern blot analysis (Gause and Adamovicz, 1995). Gene-specific primer pairs were used since they are able to detect lower levels of gene expression. RT-PCR has a disadvantage in that, quantification is difficult because many sources of various exist, including template concentration and amplification efficiency. For RT-PCR to be accurate and quantitative, it must be analyzed in the linear range of amplification before reaction components become limiting, which occurs after 20 cycles. RT-PCR, however, with 35 cycles was shown to closely resemble northern blot analysis, indicating relatively low template amount or amplification efficiency (Dean *et al*., 2002). In this study, 40 cycles were used for RT-PCR analysis, which was considered to be effective in amplifying some genes, such as *RhACS1* and

RhACS2. The number of cycles to reach saturation greatly depends on the amplification efficiency (Diaco, 1995). However, qualitative RT-PCR in this study was difficult to explain the variations in RNA levels. Marone *et al*. (2001) suggests that in most cases, a qualitative study is not sufficient to deliver a satisfactory answer for the detection of any variation in the expression levels under different experimental conditions. RT-PCR analysis showed that the expression of all genes investigated was undetectable in both 'Vanilla' and 'Lavender' petals in the absence of ethylene.

In carnation (Wang and Woodson, 1991; Woodson *et al*., 1992) and Orchid flowers (O'Neill *et al*., 1993), senescence is associated with increased ethylene production arising from concomitant increases in the activities of both ACC synthase and ACC oxidase enzymes. *ACS* gene is encoded by gene families, which are responsible for the differences in the regulation of expression among the different family members. Transcription of the *ACS* family members in *Arabidopsis* is differentially regulated during development, in different stimuli (Liang *et al*., 1992; Vahala *et al*., 1998). In this study, *RhACS1* was upregulated in 'Vanilla' control leaves by treatment of ethylene, while expression of both *RhACS1* and *RhACS2* were downregulated in 'Lavender' control leaves. The expression of *RhACS1* and *RhACS2* was also induced by ethylene treatment in 'Lavender' control petals (Fig. 16A), indicating that these genes were specifically expressed in different organs and cultivars of rose. Müller *et al*. (2000a) has shown that the ACC synthase transcript increases during flower senescence in 'Vanilla', with long-lasting flower longevity but remains constant at a low level in 'Bronze', a sensitive flower. However, *RhACS1* in rose leaves and buds is not detectable but is expressed in rose floral organs, such as ovary and sepals, and at very low level in anthers (Wang *et al*., 2004). Functionally, *ACS* genes can be classified in terms of their primary stimulus such as wounding, auxin or ripening that induces expression. The primary stimulus-induced expression of *ACS* genes is further positively or negatively modulated by secondary stimuli (Imaseki, 1999). In tomato fruit, a massive ethylene production is responsible for increases in *LE-ACS2* and *LE-ACS4* transcripts (Van Der Streten *et al*., 1990; Olson *et al*., 1991; Rottmann *et al*., 1991; Yip *et al*., 1992; Lincoln *et al*., 1993; Barry *et al*., 1996). Expression of these genes in preclimacteric tomato fruit is rapidly induced and/or enhanced by ethylene (Maunder *et al*., 1987; Rottmann *et al*., 1991; Lincoln *et al*., 1993). Additionally, expression of ACC synthase gene is proposed to be the rate-limiting step that controls the burst of ethylene occurring during fruit ripening in *Prunus mume* (Mita *et al*., 1999). In carnation flower,

expression of *CARAS1* and *CARACC3* transcripts in ovary is rapidly induced by ethylene (Have and Woltering, 1997). There might be several mechanisms that regulate the expression of ethylene biosynthesis genes during fruit ripening and flower senescence, and these mechanisms might depend, in turn, on plant species and tissue (Mita *et al.*, 1999).

Ethylene perception requires specific receptors and a signal transduction pathway to coordinate downstream response (Müller *et al.*, 2000a). Hua *et al.*, (1998) showed in *Arabidopsis* leaves that the receptor genes are differentially regulated by ethylene. The different receptors have tissue- or stage-specific functions (Chang and Stewart, 1998). Exposure to ethylene may also increase tissue sensitivity of ethylene receptors, possibly providing a mechanism to react to low ethylene concentration (Müller *et al.*, 2000a). The present results indicate that the putative ethylene receptor genes were upregulated in both 'Vanilla' and 'Lavender' petals in the absence of ethylene, while *RhETR4* transcript was not detectable in 'Lavender' control petals (Fig. 15-16A). The expression pattern of the ethylene receptor in both cultivar petals was similar to that of leaves, in which *RhETR1*, *RhETR2* and *RhETR4* transcripts were upregulated by treatment of ethylene. However, *RhETR3* was constitutively expressed in 'Vanilla' control leaves as opposed to 'Lavender' control leaves, whereby *RhETR3* was downregulated by ethylene treatment (Fig. 15-16B). This was consistent with the findings of Müller *et al.* (2000a, b) with miniature rose cultivars 'Bronze' and 'Vanilla' in which, exposure to low ethylene concentrations results in upregulation of *RhETR1* in 'Vanilla' flowers. *RhETR2* was constitutively expressed during flower senescence, while the *RhETR3* transcript in 'Vanilla' flowers appeared to be constitutively expressed at a very low level. Tieman and Klee (1999) found that expression levels of *LeETR4* and *LeETR5* transcripts in tomato are highly regulated among plant tissue with high levels of expression in productive tissues such as flower buds and mature flowers. Additionally, *Nr* gene expression can be induced by treatment with exogenous ethylene, or with treatments that induce *in vivo* ethylene production such as fruit ripening (Wilkinson *et al.*, 1995; Payton *et al.*, 1996), while other *ETR* homologues, *LeETR1*, *LeETR2*, *LeETR4* and *LeETR5*, are constant throughout the ripening process (Lashbrook *et al.*, 1998). In *Arabidopsis leaves*, transcript levels of *ERS1*, *ETR2* and *ERS2* genes are upregulated, while *ETR1* and *EIN4* are not affected by ethylene treatment (Hua *et al.*, 1998). In mung bean seedlings, ethylene positively modulates the expression of its receptor genes (Kim *et al.*, 1999). By contrast, exogenous ethylene does not induce *PhETR1* or *PhETR2* mRNA accumulation in geranium, suggesting control of ethylene-

induced petal abscission in geranium florets may lie in another member of the *PhETR* gene family or at a post-transcriptional point (Dervinis *et al.*, 2000).

Regulation of ethylene response can possibly occur downstream of the ethylene receptor. *RhCTR1-2* transcripts were upregulated after ethylene treatment in both 'Vanilla' and 'Lavender' control petals (Fig. 15A and 16A). This result was consistent with Müller *et al.* (2002). In 'Bronze' petals exogenous ethylene increases expression of *RhCTR1* and *2* and results in accelerated flower senescence (Müller *et al.* 2002). *RhCTR1-2* were constitutively expressed in 'Vanilla' leaves after ethylene treatment and *RhCTR1* was also ethylene-independent expressed in 'Lavender' control leaves. These results were consistent with the *Arabidopsis CTR1* gene that was not ethylene inducible (Kieber *et al.*, 1993; Guo *et al.*, 2003). However, *RhCTR2* was upregulated in ethylene-treated 'Lavender' leaves.

EIN3 and its homologues have been shown to act as a positive regulator of ethylene response (Chao *et al.*, 1997). The present study showed that expression of *RhEIN3* and *RhEIL* transcripts in petals and leaves of both cultivars were upregulated by treatment of ethylene (Fig. 15-16A, B). However, Müller *et al.* (2003) found that the *RhEIN3* gene was constitutively and stably expressed in petals during flower development in cultivar 'Bronze' and 'Vanilla' and in response to ethylene and ABA. Additionally, ethylene does not affect the level of *EIN3* and *EIL1* genes in *Arabidopsis* (Chao *et al.*, 1997; De Paepe *et al.*, 2004) and in tobacco (Kosugi and Ohashi, 2000). In contrast to carnation flowers, the expression pattern of *EIL1* (a homologue to *AtEIN3*) decreases during senescence, suggesting *EIN3* and its homologue may possibly be involved in the regulation of ethylene sensitivity (Waki *et al.*, 2001). However, *EIN3/EIL* transcription factors are not primarily regulated by ethylene at the transcriptional level (Chao *et al.*, 1997; Tieman *et al.*, 2001; Lee and Kim, 2003). Post-transcriptional regulation in ethylene signal transduction pathway is under regulation of a key transcription factor, *EIN3*. In the absence of ethylene, *EIN3* is continuously degraded through the proteasome-mediated pathway, and prevents activation of its transcriptional targets. In the presence of ethylene, degradation of *EIN3* is suppressed, and allows *EIN3* protein levels to increase and thus promotes the ethylene response (Guo and Ecker, 2003; Potuschak *et al.*, 2003).

The primary ethylene response element, *ERF1,* is considered as an immediate target of *EIN3* (Solano *et al.*, 1998).

The protein of the newly-isolated gene *RhERF1* contained a highly-conserved Ap2 DNA binding domain. This domain was the only part of the protein that exhibited significant sequence homology with ERF proteins from other species. The binding of some ERF proteins to the GCC box in the ethylene-responsive element suggests a role for proteins in the regulation of expression of the ethylene-responsive gene. As a transcription factor initiating the expression of related target genes, the ERF protein plays an important role in physiological plant processes (Qin *et al.*, 2004).

Both 'Vanilla' and 'Lavender' miniature rose cultivars showed that *RhERF1* was positively regulated when exposed to exogenous ethylene (Fig. 15-16A, B). In *Arabidopsis*, *AtERF1*, *AtERF2* and *AtERF5* are rapidly induced by ethylene, 12 h after treatment and their roles are to regulate the ethylene-inducible gene (Solano *et al.*, 1998). The transcripts of *GbERF* accumulate rapidly to a high level of *G. barbadense* in the leaf, when treated with exogenous ethylene (Oin *et al.*, 2004). This study indicated that all genes are specifically expressed in miniature roses and the levels of mRNAs are regulated in a tissue-specific manner (Shibiya *et al.*, 2002).

Ethylene-receptor inhibitors such as 1-MCP and CPs long-chain, are thought to act by binding to a metal in the receptor (Sisler and Serek, 2003). 1-MCP suppresses the ethylene response pathway by permanently binding to a sufficient number of ethylene receptors that are bound to the copper cofactor of the ethylene receptor. Due most likely to steric hindrance, the binding is insufficient to convert the receptors to the inactive (off) state, which keeps *CTR1* in its active (inhibiting) state (Binder and Bleecker, 2003). By analogy to 1-MCP, CPs long-chain binds to the receptor in the same way (Sisler *et al.*, 2003). Lelievre *et al.* (1997) showed that treatment with 1-MCP results in reduced accumulation of ACC oxidase and ACC synthase transcripts, and ethylene production during chilling of pear fruits. In addition, 1-MCP antagonizes not only ethylene responses but also ethylene biosynthesis, by downregulating *CsACS1* and *CsACO1* in *citrus* fruitlets (Katz *et al.*, 2004). Moreover, 1-MCP results in the elimination of mRNA levels for receptor genes of pears during the cold treatment (El-Sharkawy *et al.*, 2003). In miniature roses, pretreatment with 1-MCP obviously reduces the rise in autocatalytic ethylene production in

flowers (Müller and Stummann, 2003). In the present study, pretreatment with 1-MCP and 1-OCP showed strong suppression of the expression of all genes investigated in both 'Vanilla' and 'Lavender' petals in the absence and presence of ethylene, while *RhETR3*, *RhEIN3* and *RhEIL* were strongly expressed in 'Lavender' petals pretreated with 1-DCP in the presence of ethylene. 1-MCP, 1-OCP and 1-DCP eliminated the expression of all genes in 'Vanilla' leaves in the absence of ethylene, while *RhEIN3* transcript was upregulated after pretreatments of 1-MCP and 1-OCP in the presence of ethylene. In contrast to 'Lavender' leaves, 1-MCP and 1-OCP pretreatments prevented the accumulation of *RhEIN3* mRNA (Fig. 15-16B). The results provided evidence that at least some signal transduction genes may play an important role in ethylene perception and signal transduction pathway, and are involved in ethylene-induced flower senescence. *RhEIN3* transcript is probably rate-limiting for ethylene perception and signal transduction pathway, which is regulated during flower senescence. The expression of *RhEIN3* transcript in 'Vanilla' and 'Lavender' leaves pretreated with 1-MCP and 1-OCP may also be regulated at the transcriptional level. A physiological investigation can explain a large difference of display qualities in both miniature rose cultivars, when pretreated with 1-OCP and 1-MCP, caused the increased number of leaf drop in 'Vanilla' more than in 'Lavender' (Fig. 7A, B, C, D).

In accordance with 1-MCP, pretreatment of 1-OCP of 'Lavender' leaves reduced the expression of all genes investigated except for *RhEIL*, which was upregulated in the presence of ethylene, while 1-DCP treatment upregulated transcripts of *RhETR1*, *RhCTR2* and *RhEIN3*. This may result in plants being more sensitive to ethylene. Additionally, the abundance of the individual transcripts varied with different tissues (Lashbrook *et al.*, 1998; Tieman and Klee, 1999). 'Vanilla' was characterized by excellent longevity (Müller *et al.*, 1998), while 'Lavender' exhibited short postharvest life. Therefore, exposure to ethylene may increase the sensitivity of tissues in 'Lavender' than that of 'Vanilla', thus resulting in reduction in postharvest longevity.

It seems possible that 'Vanilla' differs from other miniature rose flowers in its ethylene-binding site or signal transduction pathway (Müller *et al.*, 1998). Even though 'Vanilla' seemed insensitive to ethylene, exogenous ethylene positively modulates the expression of ethylene receptor gene in flowers (Müller *et al.*, 1998, 2000).

Based on the genetic evidence, these results correlated with physiological investigation that 1-MCP is an ethylene-action inhibitor that binds to the receptor site competitively, thereby preventing tissue response to ethylene (Sisler and Serek, 1997). 1-MCP was more effective than any other long chain CPs in preventing flowers from exogenous ethylene. Differences between 1-MCP and CPs long chain may be due to different chemical groups attached to the cyclopropene molecule or to the side chain(s), and some of them may have interesting physiological properties (Sisler *et al.*, 2003).

In conclusion, it is known that ethylene biosynthesis, ethylene perception and the signal transduction pathway contribute to the regulation of ethylene responses in plant tissues. These results indicated both positive and negative feedback regulation mechanism exists in miniature rose cultivars 'Vanilla' and Lavender' pretreated with ethylene receptor inhibitors, 1-MCP, 1-OCP and 1-DCP, in the presence or absence of ethylene. The mechanisms that regulate the expression of genes for ethylene biosynthesis, ethylene perception and the signal transduction pathway during flower senescence might depend on plant species and tissues under investigation. 1-MCP, 1-OCP and 1-DCP treatments suppressed the expression of these genes. Therefore, an explanation of the role of the ethylene receptor inhibitors treatment in miniature roses is required to understand the mechanism of gene expression in the same response but under different conditions.

4. Summary

Ethylene-mediated flower senescence, especially in ornamental crops, is a significant problem in the horticulture industry (Müller and Stummann, 2003). Exogenous ethylene reduces longevity of commercial miniature rose cultivars (Serek, 1993; Serek *et al.*, 1994c), which showed great variation in their display life in an ethylene-free environment. Sensitivity to ethylene seems to be an important natural regulator of rose flower senescence and varies greatly among cultivars (Müller *et al.*, 2000). Therefore, developmental regulation of ethylene response in flowers may occur at the level of hormone sensitivity (Tieman and Klee, 1999).

Ethylene action involves binding to a specific receptor (Schaller and Bleecker, 1995). In the present study, the potency of two structural analogues of 1-MCP, 1-OCP and 1-DCP, containing a side chain at 1-position, was assessed. These chemicals are new putative inhibitors of ethylene action. Their potency was evaluated by testing their ability to counteract ethylene-induced responses (display life, bud, flower and leaf drop). 1-OCP and 1-DCP protected plant tissue against the detrimental effect of ethylene for a longer time as compared to untreated (control) plant, suggesting these analogues were similar to 1-MCP (Sisler and Wood, 1988; Sisler and Serek, 1997). These two analogues exerted their effect by blocking the site of ethylene binding at the receptor. The best response with 1-OCP and 1-DCP was achieved at a concentration 5 times higher than that with 1-MCP. The results of parallel experiments with 1-MCP showed that the two analogues were less potent than 1-MCP. 1-MCP improved display life by delaying bud, flower and leaf abscission for an extended period at 200 nl l[-1], whereas two analogues improved display life at 1000 nl l[-1]. However, no phytotoxicity was observed at higher concentrations of 1-OCP and 1-DCP. The time of exposure had an influence on the binding of two chemicals to the ethylene receptor, thus affecting the display life of the plants. 4-h exposure time to 1-OCP and 1-DCP treatments was sufficient to improve the display life for about 9 days. This was significantly different from untreated plants, which showed 80% leaf drop after 6 days. Continuous exposure of two analogues for a longer period of time possibly resulted in saturation of the receptor and did not give any additional benefit. However, after 9 days of pretreatment with 1-OCP and 1-DCP, the tissue became sensitive to ethylene again, indicating that free binding sites on the ethylene receptor were present in the tissue at the point of recovery from the inhibition, or these binding sites were newly formed, or the sites became dissociated from the

inhibitors (Feng *et al*., 2004). Miniature roses pretreated with 1-OCP and 1-DCP at the range of 5-10°C were not significantly different but were different from untreated (control) plants in improving display life. However, 1-MCP was more effective than 1-OCP and 1-DCP. Based on the concentrations required and the duration of protection, 'Lavender' flowers, which are sensitive to ethylene pretreated with 1-OCP, was more effective in protecting detrimental effects of ethylene than pretreated with 1-DCP. Conversely, 1-DCP pretreatment was more effective in 'Vanilla', with long-lasting flowers, than with a 1-OCP pretreatment. We also showed that 1-MCP analogues, with substitution at the 1-position and bearing eight and ten carbon side chains, are effective as blockers of the ethylene receptor.

The molecular study explained the expression of genes for ethylene biosynthesis, ethylene perception and signal transduction after pretreatment with 1-MCP, 1-OCP and 1-DCP, and afterwards continuous exposure to exogenous ethylene was performed in petals and leaves of both 'Vanilla' and 'Lavender'. It is widely recognized that ethylene biosynthesis, ethylene perception and the signal transduction pathway involve the regulation of ethylene response in plants (Wilkinson *et al*., 1995). To gain a better understanding of the ethylene response pathway, therefore, new partially-putative ethylene-responsive element-binding factors (ERF) were isolated from *Rosa hybrida*. *RhERF1* showed a similar, highly-conserved DNA-binding domain consisting of 30 amino acids and shared homology with other reported ERF-domain containing proteins.

Northern blot hybridization analysis was used to compare the results of RT-PCR. However, it was difficult to distinguish transcriptional signals of ethylene receptor, *RhETR2*, which is a high homologue to other members of the ethylene receptor family in miniature roses, such as *RhETR1* and *RhETR4*. Thus RT-PCR was utilized to compare the expression level of genes investigated in miniature potted roses. RT-PCR analysis revealed that the expression of mRNAs for many ethylene-related genes in both rose cultivars was regulated in a tissue-specific manner. Also, differential cultivars suggest they have different roles in ethylene biosynthesis enzyme, ethylene perception and signal transduction pathway. In the petals of the two miniature rose cultivars in the absence of ethylene, no expression of mRNAs for any genes in any treatments was detectable. However, the accumulation of mRNA transcripts was upregulated by ethylene in control petals. In vegetative tissues like leaves, the level of mRNAs was detectable in both untreated cultivars in

the absence and presence of ethylene indicating the ethylene signaling pathway contains both positive and negative regulator genes. This implied that some proteins serve to induce the ethylene response while others suppress it (Chen *et al*., 2005). In 'Vanilla' control leaves, *RhACS1, RhACS2, RhETR1, RhETR2, RhETR4, RhEIN3, RhEIL* and *RhERF1* transcripts were upregulated by ethylene, while *RhETR3* and *RhCTR1-2* were constitutively expressed. In 'Lavender' untreated leaves, *RhACS1, RhACS2* and *RhETR3* transcripts were downregulated, while *RhETR1, RhETR2, RhETR4, RhCTR1-2, RhEIN3, RhEIL* and *RhERF1* transcripts were upregulated by ethylene. Pretreatment with ethylene receptor inhibitors, 1-MCP, 1-OCP and 1-DCP, suppressed the expression of all genes investigated in 'Vanilla' petals, in the absence or presence of ethylene, while pretreatment with 1-DCP upregulated the expression of *RhETR3, RhEIN3* and *RhEIL* transcripts in 'Lavender' petals in the presence of ethylene. In 'Vanilla' leaves, in the absence of ethylene, all chemicals suppressed the expression of all genes investigated, whereas the level of *RhEIN3* mRNA was upregulated by pretreatment of ethylene receptor inhibitors in the presence of ethylene, while the accumulation of *RhEIN3* mRNA was suppressed in 'Lavender' leaves. These results suggest that the expression of *RhEIN3* transcript in 'Vanilla', which is insensitive to ethylene, and 'Lavender', with short-flower life, may be rate-limiting for ethylene perception and the signal transduction pathway that is involved in ethylene-induced flower senescence. With physiological investigation, this can explain a great difference of display quality between 'Vanilla' and 'Lavender' when pretreated with 1-OCP and 1-MCP. Pretreating 'Lavender' leaves with 1-MCP and 1-OCP in the presence of ethylene suppressed the expression of other genes but upregulated the accumulation of *RhETR1, RhETR2* and *RhEIL* transcripts, respectively, while pretreatment of 'Lavender' leaves with 1-DCP upregulated *RhETR1, RhETR4, RhCTR2, RhEIN3* and *RHEIL* transcripts.

The regulation of genes for the ethylene biosynthesis enzyme, ethylene perception and signal transduction pathway in miniature rose is partly due to the differences in sensitivity of cultivars and tissues to ethylene. Exposure to ethylene may increase the sensitivity of tissues in 'Lavender' than that of 'Vanilla', thus resulting in reduction in postharvest longevity. This might depend on plant species and tissue. However, 1-MCP was more potent in protecting plants against ethylene than 1-OCP and 1-DCP, probably by blocking the binding site of ethylene on the ethylene receptor in plant tissues, thus controlling ethylene responses (Sisler and Wood, 1988; Sisler and Serek, 1997). 1-MCP is a highly strained olefin, which binds in an apparently irreversible manner

to the ethylene receptor. Also, it seems that 1-OCP and 1-DCP inhibited ethylene action in a similar mode to that suggested for 1-MCP. These results were consistent with physiological investigation except after a certain period, when the plant tissues resumed sensitivity to ethylene (Sisler and Serek, 1997).

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6. Appendices

Appendix 1. gene-specific primer pairs for *RhERF1* using partial DNA fragment of *Arabidopsis thaliana*

TACAGAGGCGTTAGGAAGCGTCCTTGGGGCCGTTATGCCGCCGAGATCCGAGATCCGGGCAAGAAAACCCGCGTCTGGCTTGGCACTTTCG

Appendix 2. Sequence analysis of *Rosa hybrida* 92 bp partial DNA fragment. (a) nucleotide sequence, (b) mRNA sequence, (c) deduced amino acid sequence (d) homology comparison with *LeERF1* (GeneBank Accession No. AY192367), *LeERF2* (GeneBank Accession No. AY192368), *AtERF1* (GeneBank Accession No. AAL25588), *AtERF2* (GeneBank Accession No. NM_124093) and *AtERF5* (GeneBank Accession No. NM_124094) using CLUSTAL W program. [*]= identical amino acid, : = two nucleotides out of the triplicate amino acid code are identified, . = one nucleotide out of the triplicate amino acid code are identified.

(a)

CGAAAGTGCCAAGCCAGACTCTCGTCTTCTTCCACGGGTCTCTGATCTCGGCGGCGTAGCGACCCCACGGCCGCTTCCTAACGCCTCTGTAA

(b)

CGAAAGTGCCAAGCCAGACTCTCGTCTTCTTCCACGGGTCTCTGATCTCGGCGGCGTAGCGACCCCACGGCCGCTTCCTAACGCCTCTGTAA

(c)

YRGVRKRPWGRYAAEIRDPWKKTRVWLGTF

(d)

```
LeERF1   106   YRGVRQRPWGKFAAEIRDPAKNGARVWLGTYESAEEAALAYGKAAFRMRGTKALLNFPHR 166
LeERF2    60   YRGIRQRPWGKWAAEIRD-PRKGVRVWLGTFNTAEEAARAYDREARKIRGKKAKVNFPNE 119
AtERF1     2   YRGVRKRPWGKYAAEIRDSARHGARVWLGTFNTAEDAARAYDRAAFGMRGQRAILNFPHE  62
AtERF2   116   YRGVRQRPWGKFAAEIRDPAKNGARVWLGTFETAEDAALAYDIAAFRMRGSRALLNFPLR 176
AtERF5   155   YRGVRQRPWGKFAAEIRDPNKRGSRVWLGTFDTAIEAARAYDEAAFRLRGSKAILNFPLE 215
RhERF      1   YRGVRKRPWGRYAAEIRDPWKK-TRVWLGTF---------------------------  30

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91

Appendix 3. Sequence analysis for specific primers of *Rosa hybrida* (a) nucleotide sequence, (b) mRNA sequence. Border between intron and exon sequence was detected with splicing specific sequence <mark>GT</mark> and <mark>AG</mark>, respectively.

β-Actin: X55751   373 bp mRNA

(b)

β-Actin_s CCATGTTCCCTGGTATTGCT
β-Actin_as TAGATGTGGATTGCAAAGGC

mRNA size 150 bp

```
ACAACTCTATCATGAAATGTGATGTGGATATTAGAAAAGATCTTTATGGAAACATTGTGCTCAGTGGTGGTACTACCA
TGTTCCCTGGTATTGCTGATAGAATGAGCAAAGAAATTACTGCATTGGCTCCTAGCAGCATGAAGATTAAGGTGGTCG
CTCCACCAGAGAGGAAATACAGTGTCTGGATTGGAGGCTCTATCTTGGCTTCCCTCAGCACCTTCCAGCAGGTCTCGT
CGCCCCTTCCCCTTCCCCCTCCCTCCTAATAATTATAAGTTTGCTTTTTTTCGGTGTCTATTGTACTTCGAAGCTTGA
CAGTTACTGACGGTCTTTTCTGCTTCTCTTATTTTCTGTAGATGTGGATTGCAAAGGCAGA
```

RhACS1: AY061946   1089 bp mRNA

(b)

RhACS1s GCCACTGGAGCTAACGAGAC
RhACS1as TGTTTCGGGCCTTTTCATAG

mRNA size: 205 bp

```
ATGGGTCTGGCGGAAAATCAGCTTTCTTTCGACGTGATTGAAGAGTGGATTAAGAAAAATCCCAAGGCCTCCATTTGC
ACTCCTGAAGGAGTTGCGGAGTTCAAAAATGTAGCCAACTTTCAAGACTATCATGGCTTTCCAGACTTCAGAAAGGCT
ATTGCTAACTTCATGTCAAAGGCGAGAGGAGGCAGAGTCAAATTTGATCCTGATCGCGTAGTCATGGCCGGCGGAGCC
ACTGGAGCTAACGAGACGGTCATGTTTTGCTTGGCTGACCCCGGAGATGCTTTCCTAGTTCCCTCTCCTTACTATCCA
GCGTTCTACCGAGACTTGGGGTGGCGAACGGGAGTGAAAATTGTTCCAGTCGATTGTGATAGCTCAAACAATTTCCAA
GTGACCAGAGCAGCACTTGAAGCAGCCTATGAAAAGGCCCGAAACAACAACATCAACATAAAGGGCTTGATCATAACA
AACCCTTCAAACCCATTGGGGACAACCGTAGACAGAGACACACTCACTAGCTTGGTCAAATTCATCAATGACAAGAAC
ATCCACTTGGTCTGCGACGAAATATACGCAGGCACAGTGTTCAGCTGCCCAAAATTCACTTGCGTTACAGAGGTCCTA
CAAGACGTGAAGAATTGCAACCGCGATTTGATTCACATTGTCTATAGCTTGTCCAAGGACATGGGTGTCCCCGGTTTA
AGGATCGGGATCGTTTACTCCTACAACGACGCCGTCGTGAACTGCGCTCGCAAGATGTCAAGTTTCGGCTTGGTCTCC
TCCCAAACTCAACACATGCTCTCCGCCATGCTTCTGGACGACGAATTTGTCTCCAACTTCCTTGAGACCAGCTCTAAG
AGGCTGGCGAGGAGGCACCGGTTTTTCACCACGGGGCTCGAGGAGGTAGGCATCAACTGCTTGAAAGGCAATGCCGGG
CTCTATTGCTGGATGGACTTGAGGAAGCTGCTCAAAGACCAAACTTTCGAGGCGGAAATGGTGCTGTGGCGTATGATT
ATCAACGAAGTGAAGCTCAACGTTTCGCCGGGGTCTTCCTTCCGTTGCGTGGAGCCAGGCTGGTTCCGGGTTTGT
```

RhACS2: AY525066   1346 bp DNA

(a)

RhACS2s   AAAACCCAGAAGCCTCCATT
RhACS2as ATTTCTGGTTCCCGTTCCTT

Genomic size 369 bp

```
ATGGGGCTGGCGGAAAATCAAGTAAGAGATTCAAAGACAGAAACCTTGTTTTACAAGCATAATTTCATCTCTTGGCTA
TTTACTAATTGGTTTCTTCTGTATGCAGCTTTCTTTTTGGTTTGATTCAAGAATGGGTTCTGAAAAACCCAGAAGCCTC
CATTTGCACTGCACAAGGAGCAAAAGCATTCAAGGACATAGCCATCTTTCAAGACTATCATGGCCTGCCAGAGTTCAG
AAATGTATGTATTGATCAACATCTACAAGATTTTCTTAGCATCACTAATTAACTCATTACACAATCTTACCAAGTTTG
TTGTTCAAATATTAACAAAGTTAATTATTGTTGTGAAACTCCACAGGCTGTTGCAAATTTCATGGGAAAAGTGAGAGG
AAATCGAGTCACATTCGACCCTGACCGCATTGTTATGAGCGGCGGAGCAACCGGAGCTCACGAGATGATTGCCTTTTG
CTTGGCTGATCCCGGAGAGGCATTTCTGGTTCCCGTTCCTTATTATCCTGGGTAAGCTTAATTTTATATATCTTTCTA
AGATTGACTATGATCCAACTAAGACGGGAAAAATTAGCTAATTAATTTAGGCGAGTTGCCTAATATTTTATGAGAAAT
TAGCAATGAAAGAGACTGGTGTTGACCCTTATGTGGTCTTGGACCATCTTGAATTATTGAGGGGGGAATCAAAACGAT
CTTTGTACAAATTGTCTTGGCTAGACACCATTCAATCTATACCTCACTTTGATAAACATGAAAGATGCCTTGGGAAAA
TAAGGACCATCCTTCCTTTTAAAAGGACAAGTAAAACTGTACAATTGGACACTTGTAAACTTCTTATGGTAGCTAGGC
GCACTGATCACGTCATTAAAATATATTCTTTATGGAGATCTGACAAGTAGATTATCTACTTTGATACTTATTTATTGT
TATGCGAAATTTAGAATTCTCGACGTAGAATTCCATATTTATTCATAACGTACGGTTAAAATATTCTCCATGCATGAG
TTGCTTATAAGTAGATTATCTACTCTGATACTTATTTATTGCTATGCGAAATTTAGAATTCTCGACGTAGAATTTCAT
ATTTGTTTACAACGTACGGTTAAAATATTCTCCATGCATGAGTTGCTTGCGAAATAACTTTTTTACATCATTGCAGAT
TCGATAGAGATTTGAGATGGCGAACAGGGGTACAACTTCTTCCAGTTGCTTGTGAAAGCTCCAACAATCTCAAGATCA
CCAGAGCAGCCTTGGAAGATGCCTATGAGAAAGCCCAAAAGGCCAACATCAGAGTTAAAGGCTTACTCATTACCAACC
CCTCCAACCCCTTCGGCACA
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(b)

mRNA size 249 bp

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ATGGGGCTGGCGGAAAATCAAGTAAGTTTTTGGTTTGATTCAAGAATGGGTTCTGAAAAACCCAGAAGCCTCCATTTGC
ACTGCACAAGGAGCAAAAGCATTCAAGGACATAGCCATCTTTCAAGACTATCATGGCCTGCCAGAGTTCAGAAATGCT
GTTGCAAATTTCATGGGAAAAGTGAGAGGAAATCGAGTCACATTCGACCCTGACCGCATTGTTATGAGCGGCGGAGCA
ACCGGAGCTCACGAGATGATTGCCTTTTGCTTGGCTGATCCCGGAGAGGCATTTCTGGTTCCCGTTCCTTATTATCCT
GGGTTCGATAGAGATTTGAGATGGCGAACAGGGGTACAACTTCTTCCAGTTGCTTGTGAAAGCTCCAACAATCTCAAG
ATCACCAGAGCAGCCTTGGAAGATGCCTATGAGAAAGCCCAAAAGGCCAACATCAGAGTTAAAGGCTTACTCATTACC
AACCCCTCCAACCCCTTCGGCACA
```

RhETR1: AF394914   1479 bp mRNA

(b)

RhETR1s   TGGTATGAACCTTCAACTTTCTCA
RhETR1as CGCATAGACTCTTCAAGAATAGCA

mRNA size: 393 bp

```
GAAACCACTCTTGTTGAGCTGGGAAGGACTTTAGGCCTTGAGGAATGCGCTTTGTGGATGCCGTGCAGGAGTGGTATG
AACCTTCAACTTTCTCATACTCTCAACTATGAAGCACAAATTGGGTCTACTGTGCCATTTAGCCTTCCTGTAGTTAGT
GAAGTTTTCAAGAGCTCTCAAGCAATGCGTATACCATATACCTGCCCCTTGGCCCGGGTCAGACCACTTGTGGGAAGA
TATGTGCAACCTGAGGTTGTTGCTTTGCGTGTACCGCTGTTGAATCTTTCAAATTTCCAAATCAACGACTGGCCTGAT
GTCTCTGCGAAAAGCTATGCCATCATGGTTCTGATTCTACCAACAAATAGCACTAGAAAGTGGCGAGACCATGAACTT
GAACTGGTTGATGTTGTTGCCGATCAGGTCGCTGTTGCTCTTTCACACGCTGCTATTCTTGAAGAGTCTATGCGAGCC
AGTGATCAGCTCGTAGAGCAGAATGGTGCTTTAGATTTGGCCCGGAGAGAGGCAGAGCTGGCAATCCATGCTCGCAAC
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93

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GACTTCCTTGCTGTCCTGAACCATGAAATGAGGACACCAATGCATGCAATTATTGCATTGTCTTCACTTCTTTTAGAA
ACGGACCTTGAAGATGGCAGTCTAGACTTGGACATTGGAAAGTTTAACCTCCATGGAGTTTTCAGAGAGGCAATCAAT
TTAATAAAGCCTATTGCAACTGTGAAGAAGTTATCTATGACTTTAATTCTGGCCCCAGATGTGCCTGTGTGTGCTGTT
GGAGATGAGAACCGGCTTATGCAAACTATTTTAAATGTTGCTGGTAATGCTGTCAAGTTTACAAAGGAGGGTTATATT
TCAGTTATAGCATCTGTTGCAAAACCAGATTCTTCAAGAGATTGGCGACCGCCTGAATTTTATCCAGCGTCAAGTGAC
GGCCATTTTTACCTACGAGTTCAGGTTAAAGATTCTGGCAGTGGTATTCTTCCACAAGATATTCCTCATATTTTCACC
AAATTTGCTGAGCCCAGAAATGGATCCAATCGAGTAAAAGATGGTGCAGGACTTGGACTTGCCATTTGCAAACGGTTT
GTGAATACCATGGGAGGTCATATTTGGATCGAGAGTGAGGGCATTGACAAGGGGACCCTTGTTGTATTCATCGTCAAA
CTCGGGATATGCGACAATTGCGGTAATACAACAATAGCACATCAAAAAGTACCCAAAGGGGAGGCAAATCATGGAAGT
GCAGATCTCATTGGATACAAACCAGCATTTAGAGATGGTTACGGTTTAGCTTCAACAAATCCGCGTTATCAAAGAAGT
CTTTGACTGCAAAAGTCTTTGGCTGCAATAACCGTAAATGCTGTGCTAAAAAAGAGAGATTTCCTGGAGTATGGAATG
TAGATTTTAGTAGAAGAAATTTGCGGTTTATATGATTTTATCATTTGCCTGAGTTCAAAAAAAAAAAAAAAAAAAA
```

RhETR2: AF127220 534 bp DNA

RhETR2s CTCAAACTTCCAAATCAATGACTG
RhETR2as ATATTCTGCTCCATTAGCAGATCC

(a)

Genomic size: 978 bp

```
GAGTGCGCATTGTGGATGCCCACGCGTACTGCGTTAGAGCAGCAACTGTCGTACACACTCCATCAGCAGAATCCGGTG
GGACATACCGTACCCATTCATCTTCCTGTGATCAATCAAGTGTTTAGCAGCAACCATGCCGTGAAGATATCGCCGAAT
TCCCCAGTGGCAAGACTGAGGCCACGTGCGGGAAACCATATGCCTGGAGAGGTTGTTGCTGTTCGTGTCCCTCTCCTG
CATCTCTCAAACTTCCAAATCAATGACTGGCCTGAGCTCTCAACAAAACGCTATGCTTTGATGGTGTTGATGCTCCCT
TCCGATAGTGCAAGGCAATGGCATATGCATGAGTTAGAGCTGGTTGAGGTAGTTGCTGACCAGGTATGGAACCACAAT
GTTGATCATTTTCTTTTTGACATCATTAGTTATCACCCAATGCCTAAATTGCAAAAAGAAAAAGAAAAAAACAAT
TTTTTTTATACAACTCTCCTTTATGTTATTGTTTGTCTCTTTCTTAAGATGGTTACTTTATGTATTCATAATAATAAA
TTTTTTTATTGTTTATGACATTCTTCATTCCTTTGGCAGTCACTTGCAATAGGAATTCTCAGGATATCCCAGCGTCTA
GGTTGTCTTATTATGAAAATGAAAGTGTGTGAATCATAAGTAATTGTCGATCTGCAAAATGGTGGATCTATGCTCTG
TCATTTTATTTTATATTCACAAGTCTGCAAGTAGACATAAATAGTAATCCTCACTAACTAGTATTTACTGGTCAAATG
TCCAGATACTTTTAGAGGAGGGCTGGGACCCTAAAACCTTCTGAGTAGGCTGCAACTTTTCCTTTTTTTCTGATTCTTA
TACAACAGCCCTTGGATTAGGATGAAGTTACGTTGGCTCTTAGGAAACTATCATATAATGTAGCTTGATTGTCGCAAG
CATGAACCTTTATAGATGCCTTGCTTATAATAAAAGAAAACAAAAATGTCTTGCAGTACTTAGTGGATCTAATGAGAA
CAAAACATACATAATCATCTTATAATAACTGAACACATATACTATCTGTTTATTGCTTGCTTCATTAGTAATGGTTGC
TTATAATAAGAAGCATCAATTTATTTAATCTTATGAATTATGATACAGGTAGCAGTTGCTCTATCACACGCTGCTATC
TTAGAAGAGTCAAGGAGAGCGAGGGATCTGCTAATGGAGCAGAATATTGCACTTGATCTGGCTAGGAGGGAAGCAGAA
ACGGCAATTTGTGCTCGCAATGATTTCTTGGCCGTGATGAACCACGAGAT
```

(b)

mRNA size: 213 bp

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GAGTGCGCATTGTGGATGCCCACGCGTACTGCGTTAGAGCAGCAACTGTCGTACACACTCCATCAGCAGAATCCGGTG
GGACATACCGTACCCATTCATCTTCCTGTGATCAATCAAGTGTTTAGCAGCAACCATGCCGTGAAGATATCGCCGAAT
TCCCCAGTGGCAAGACTGAGGCCACGTGCGGGAAACCATATGCCTGGAGAGGTTGTTGCTGTTCGTGTCCCTCTCCTG
CATCTCTCAAACTTCCAAATCAATGACTGGCCTGAGCTCTCAACAAAACGCTATGCTTTGATGGTGTTGATGCTCCCT
TCCGATAGTGCAAGGCAATGGCATATGCATGAGTTAGAGCTGGTTGAGGTAGTTGCTGACCAGGTAGCAGTTGCTCTA
TCACACGCTGCTATCTTAGAAGAGTCAAGGAGAGCGAGGGATCTGCTAATGGAGCAGAATATTGCACTTGATCTGGCT
AGGAGGGAAGCAGAAACGGCAATTTGTGCTCGCAATGATTTCTTGGCCGTGATGAACCACGAGAT
```

RhETR3: AF154119   798 bp mRNA

(b)

RhETR3s   CACTGCTATAACGCTCATCACTCT
RhETR3as CATTAGTTGGGACTCTTCAAGGAT

mRNA size: 661 bp

TTTATCGCGTTTATCGTGCAATGTGGAATGACACATTTGCTGAATGGCTGGACTTATGGTCCTCACCCTTTTCAGCTA
ATGCTTGCTCTCACAGTTTTCAAAATTCTCACTGCTCTGGTATCATGTGC<mark style="background-color:#cccccc">CACTGCTATAACGCTCATCACTCT</mark>CATT
CCTTTGCTTCTCAAGGTCAAAGTGAGAGAATTTATGTTGAAGAAGAAGACTTGGGACCTTGGAAGAGAGGTTGGGATT
ATAATGAGACAGAAAGAAGCTGGAATGCATGTTCGAATGCTTACCCAAGAGATTCGCAAGTCTCTTGATAGACATACA
ATATTGTCCACAACCCTCTTTGAGCTATCTGAGACATTGGGTTTGCAGTACTGTGCAGTTTGGATGCCTAATGAAATT
AAAACGGAGATGATCCTGACCCATGAGTTGAAAGGGAGGAATTATTCTAATATGTACAACTTTTCTATACCAATAGGT
GATCCAGATGTTGCACTTATCAAAGGGAGTGATGGGGTCAACATCCTTGGGCCAGATTCAATACTCGTGTCCGGAAGC
AGTGGTGATTTTGGTGAGCCGGGACCAGTAGCTGCAATACGGATGCCAATGCTTCGGGTTTCCAATTTCAAAGGGGGG
ACCCCTGAGTTCATCCAGACTTGTTATGCGATTTTGGTTCTGGTTCTCGCTGGTGGACAGCCTAGATCTTGGAGCAGC
CAGGAACTTGAGATAATTAAGGTGGTTGCCGATCAGGTGGCTGTGGCTTTATCCCATGCTGCA<mark style="background-color:#ff00ff">ATCCTTGAAGAGTCC</mark>
<mark style="background-color:#ff00ff">CAACTAATG</mark>CGCGAGAAA

RhETR4: AF159172   534 bp mRNA

(b)

RhETR4s   TTTGAATCTGCAACTTTCTCACAC
RhETR4as GCATTTCGTGGTTCATGACAG

mRNA size: 500 bp

GAATGTGCTTTTTGGATGCCATCGCGAACGGG<mark style="background-color:#cccccc">TTTGAATCTGCAACTTTCTCACAC</mark>CCTAACCCAGGTATCGTCGGGA
GCTAATGTGTCAATAAACCTCCCAATAGTCACTGAGGTTTTCAACAGTGCCCGAGCATTGCGTATACCATATACATGC
CCGTTGGCTAGGTTTAGACCTCTTGTTGGGCGGTATATGTCACCGGAGGTTGTTGCTGTGCGAGTACCGCTTTTAAAT
CTCTCGAACTTCCAAATTAATGATTGGCCTGAACTCTCTGCGAAAAGCTATGCTATAATGGTTCTGATTCTTCCTACG
GATAGTGCAAGGCAATGGCGGGAACATGAGTTGGAACTTGTTGAAGTCGTTGCAGACCAGGTCGCTGTTGCTCTTTCA
CATGCCGCTATTCTGGAGGAGTCCATGCGTGCCCGTGATCAGCTCATGGAGCAGAACGTTGCTCTAGATTTAGCTCGA
AGAGAAGCTGAGATGGCAATCCATGCTCGCAACGACTTCCTTG<mark style="background-color:#ff00ff">CTGTCATGAACCACGAAATGC</mark>GA

RhCTR1: AY032953   3121 bp mRNA

(b)

RhCTR1s   GGCTCTGATGTTGCTGTGAA
RhCTR1as TCAATGGCCTCAAAGATTCC

mRNA size:706 bp

ATGGAAATGCCCGGTAGGAGATCGAACTACACGCTTCTCAGCCAAGTCCCCGACGATCACTTCGCTGCGGCGACGGCG
ACGTCGTTTTACGAGTCGGAGGGGAAGAACAACAATAATAAGGCGAAGGGGGATAGCAGAGGGTTTGATTGGGAAACG
GGAGGCGGCGAGTACCGGGCGGCGCCGGCGAATCGGATCGGAAACGTGTACTCGTCGGTCGGATTGCAGCGGCAGTCG
AGCGGGAGCAGCTTCGGAGAGAGCTCGCTGTCCGGCGAGTACTACGCGCCGACTCTGTCGACCACGGCGGCGAATGAG
ATTGACGGATTCGGGTACGTCAACGACGACGGGTTTAAAACCGGCGGAGGAGGCGGTGAGTTCAGAGGGAAAGGAGGA
GGGATGGATGGGGGTGTGGGCCCCCCCGGAGGTTCGTCGTCGGGGAAGAGCTGGGCGCAGCAGACTGAGGAGAGTTAT
CAGCTGCAGTTGGCTTTGGCGCTTCGGCTCTCGTCGGAAGCCACTTGCGCCGATGATCCTAATTTCTTGGATCCAGTG
CCGGATGAATCTTCGTCGAGGTTGTCCAGCTCTGCCGATGCAGTTTCGCATCGATTTTGGGTGAATGGCTGTCTGTCA

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TACTTTGACAAAGTGCCTGATGGGTTTTACCTTATTCATGGGATAGATTCGTATGTATGGTCTATGTGCACTGATGTG
CAAGAGAGTGGTCGTATACCATCAATTGAATCACTAAAATCTGTTGATCCTGGTACTGGATCTTCCATAGAAGTGGTC
TTAATCGATCGACGTAGTGATCCCAGCTTAAAGGAGCTTCAAAATAGGGTCCTCAGCATTTCTTATGCTTGCATAACC
ACGACAGAGATTGTTGATCAGCTGGCAAAGCTTGTATGCAGCCGTATGGGGGGGTTCAGCATCTGTAGGAGAGGCTGAA
TTTTTCTCCATATGGAGGGAGAGCAGTGATGATCTAAAAGATTGCTTAGGATCTGTAGTTGTTCCAATCGGCAGCCTT
TCTATTGGCCTCTGTAGACATCGTGCTTTATTATTCAAGGTTCTAGCTGACACAATTGACCTACCGTGCCGAATTGCC
AAAGGCTGTAAATATTGTACAAGAGATGATGCTTCCTCTTGTCTTGTTCGTTTTGGTATTGACAGGGAATTGTTAGTT
GATTTGATTGGGAATCCAGGTTGCTTATGTGAGCCTGATTCCTTGCTCAATGGTCCATCATCCATCTCAATTTCTTCA
CCGCTGCGATTTCCAAGACTCAGAACAGTTGAACCTACCATTGATTTCAGGTCACTGGCCAAACAGTACTTCTCGGAC
TGTCAGTTGCTTAATCTTGTATTCGATGAAGCTCCGGCAGGAAGTGCTGGTGATGAGGACAATAAAGGATTCTCTATG
TATCCTAAGCAGAAGTTTACAGATGGAAACAACCTCTTTCTGGTTTCAGGTCTTGGTGATGACACTTCTATGCATGTT
GACGATCGGAATCCCCAGTTCCTTAAATCATTTAATCCTTCTCAGAACATTGTACACCAACAAACAGTATTAAAAGAT
CAAATCCCCCTAAAGCGTATACCACCTATAGGACATAGAGATATTTCAAGGTTGGATACAAGTAAGGACTCGAGGTTT
GGTGAGGGACTTCAAGTGGTTCCTAGTAAACCAAATAAAGAGCTGACTCTCGATGTGGATGATTTGGACATTCCATGG
AGTGACCTTGTACTGAAGGAGAGAATCGGTGCAGGTTCTTTTGGAACTGTTCATCGTGCTGATTGGCAT‹GGCTCTGAT
GTTGCTGTGAA›GATTCTTATGGAGCAAGAATTTCATGCTGAACGCTTTAATGAGTTCTTGAGGGAGGTTGCAATTATG
AAACGCCTGCGGCATCCAAATATTGTGCTGTTTATGGGTGCAGTTACAAAGCCCCCAAACTTGTCCATAGTCACAGAA
TATCTATCAAGAGGGAGCTTGTATAGGCTGTTGCATAAACCTGGCCCAATACTGGATGAGAGGCGCCGGTTGTATATG
GCTCATGATGTGGCAAAGGGGATGAATTATCTTCATAGGCGGAATCCTCCCATTGTTCATCGAGATTTAAAATCACCA
AACCTTTTGGTTGACAAAAAGTATACTGTGAAGGTTTGTGATTTTGGTCTTTCCCGATTAAAGGCCAACACCTTTCTT
TCATCAAAATCAGCTGCAGGGACTCCTGAATGGATGGCACCAGAAGTCCTGCGTGATGAACCATCAAATGAGAAGTCA
GATGTTTACAGTTTTGGTGTAATATTGTGGGAACTTGCAACATTGCAACAGCCCTGGGGTAATTTGAATCCAGCGCAG
GTTGTGGCAGCTGTTGGTTTTAAGAACAAAAGGCTTGAGATTCCACGTGATTTGAATCCTCAAGTGGCTTCTATAATT
GAGGCTTGCTGGGCCAATGAGCCCTGGAAACGACCTTCATTTGCTAGTATCAT‹GGAATCTTTGAGGCCATTGA›TTAAA
GCTCCCACACCTCAACCTAGTCATGCAGACCGGAGTGGATGGCGCCAGAAGTCCTGCGTGATGAACCATCAAATGAGA
AGTCAGATGTTTACAGTTTTGGTGTAATATTGTGGGAACTTGCAACATTGCAACAGCCCTGGGGTAATTTGAATCCAG
CGCAGGTTTGTCTTTTAAGCAATATGATGAATGTTAACACCCATACATACAACATTCAGTGATGTTGCTCTTATAGAT
TAGAAAGAATATCTACAAGAGCTGATGGCTATAATAACGCTTCCAAATAATTTTTTACAGTTCTTCATTTGAGAATAT
TACAGAATGGTGTTTCTCGTATTACCCTTGCTTGAACTCTTATACCCTGATGATATTCAGTTTTTCTATACAACGTTG
TAGGTTGTGGCAGCTGTTGGTTTTAAGAACAAAAGGCTTGAGATTCCACGTGATTTGAATCCTCAAGTGGCTTCTATA
ATTGAGGCTTGCTGGGCCAAGTGAGTTTTAGATTCTAATTTGTATATTAAACTACACTCCTCAAATAACTACTTTTTC
ATTAACAACAAAGATCATGCTGCAGTGAGCCCTGGAAACGCCCA
```

RhCTR2: AY029067   681 bp mRNA

(b)

RhCTR2s   TTCCTTCCAAGGGGAAGTCT
RhCTR2as CCCACTCCAAGCCAATTTTA

mRNA size: 375 bp

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GAAGTGTATCATGCGGACTGGAATGGCACGGAAGTTGCTGTGAAGAAGTTCCTAGACCAGGATTTCTCGGGTGCTGCT
TTGGCTGAGTTCAAAAGAGAAGTACGGATAATGCGTAGGCTGCGCCATCCAAATGTTGTGCTTTTTATGGGTGCTATT
ACGCGTCCTCCTAACCTCTCTATCATTACAGAG‹TTCCTTCCAAGGGGAAGTCT›ATATCGGATTATCCATCGTCCTCAT
TGTCAAATTGAAGAGAGACGAAGATTAAAGATGGCTCTTGATGTGGCAAGGGGTATGAATTGCTTGCATTCGAGCAAC
CCCACTATTGTTCACCGGGATTTGAAATCTCCAAATCTATTGGTTGATAAGAACTGGAATGTGAAGGTAGGTGATTTT
GGACTGTCGCGCTTGAAACATAACACTTTCTTGTCCTCCAAATCAACTGCTGGAACGCCTGAGTGGATGGCACCAGAA
GTTTTGCGCAACGAAAACTCAAATGAAAGTGTGATGTATATAGCTTTGGAGTAATTTTGTGGGAACTTGCTACTC‹TA
AAATTGGCTTGGAGTGGG›ATGAACCCGATGCAAGTTGTGGGTGCGGTGGGTTTCCAGAATCGCCGCCTTGACATCCCT
AAAGAAGTTGATCCCCCTGTTGCAAGGATAATTTGGCAATGCTGGCAAAACGACCCC
```

RhEIN3: AF443783   553 bp mRNA

(b)

RhEIN3s CCCTGCAGCCATAGACAAGT
RhEIN3as ACCCTGATTTCATCCACCAA

mRNA size: 236 bp

```
ATGCTGAAGATGATGGAGGTGTGTCAAGCTCGTGGATTTGTGTATGGTATCATTCCTGAGAAGGGCAAGCCAGTAAGC
GGTGCTTCTGATAACATCAGAGCATGGTGGAAAGAAAAAGTGAAGTTTGATAAGAATGGCCCTGCAGCCATAGACAAG
TATGAAGCAGAGATTCTTGCCATGACTGATGCAGACAATAACCGAAATGGTAATTCTCATACCATCCTCCAAGATCTA
CAAGATGCAACTCTTGGTTCTCTACTATCTGCATTGATGCAACATTGCGACCCCCCTCAAAGGAAGTATCCATTAGAA
AAGGCAGTTCCGCCTCCTTGGTGGCCGACAGGAAATGAAGATTGGTGGATGAAATCAGGGTTACCCTGTGGTCAGAGT
CCTCCTTATAAGAAGCCACATGACTTAAAGAAGATGTGGAAAGTTGGGGTGTTAACAGCTGTGATAAAGCACATGTCC
CCTGATATTGCAAAGATAAGGCGGCATGTCCGTCAGTCAAAATGCTTACAGGATAAGATGACTGCCAAAATCACTAGT
GAATTCT
```

RhEIN3-like: AY052825   407 bp mRNA

(b)

RhEILs TCCCTGGTTTGATGGAAGAC
RhEILas GAGGCCACCATTCCTCATTA

```
ATGATGGAAGTGTGTCGAGCTCAGGGTTTTGTTTATGGGATTATACCTGAAAAAGGAAAACCAGTGAGTGGTGCCTCT
GACAATCTTCGAGAATGGTGGAAGGACAAAGTAAGATTTGATCGTAATGGCCCAGCTGCCATTTCCAAATATCAGGCA
GATAATTCAATCCCTGGTTTGATGGAAGACTGCATTTCAGTGGCATCCACTCCACACACCCTGCAGGAGCTCCAGGAC
ACCACTCTTGGTTCACTTTTGTCAGCCCTGATGCAGCACTGTGACCCACCCCAAAGGCGTTTCCCATTGGAGAAGGGT
GTTTCCCCACCATGGTGGCCAACTGGTAATGAGGAATGGTGGCCTCAATTGAACTTGGCCAATCAGGGACCTCCCCCC
TACAAAAAGCCCCACGG
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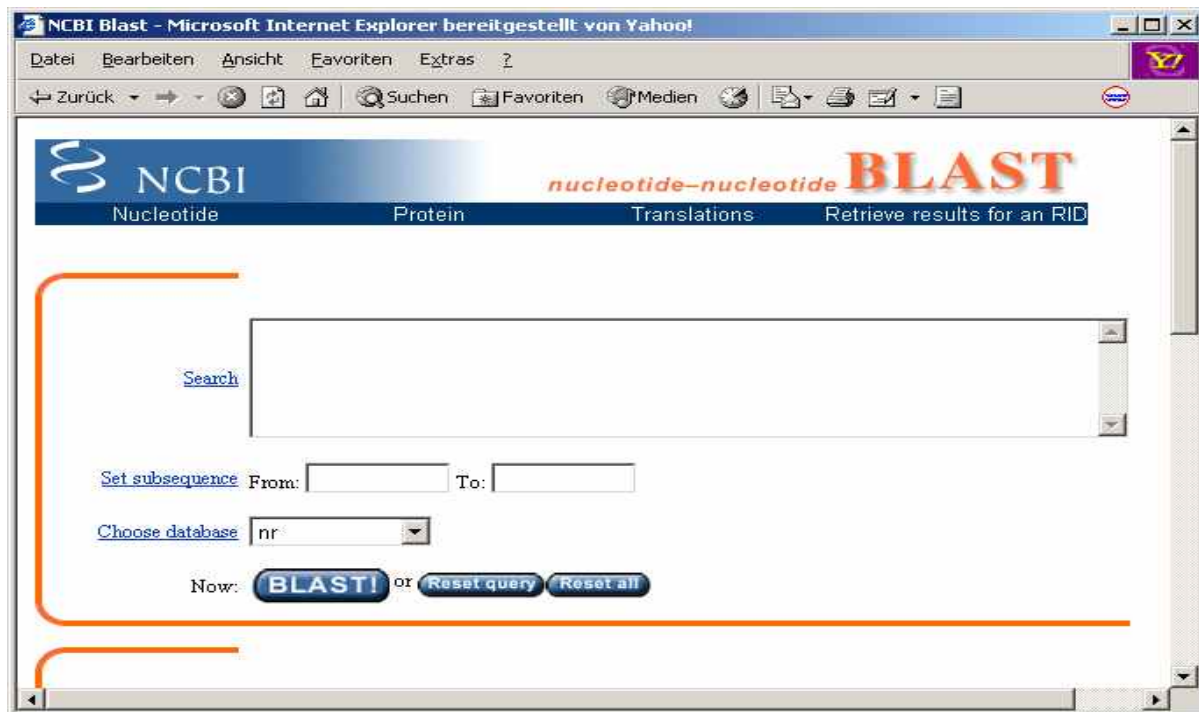
RhERF: 92 bp DNA

(a, b)

RhERFs CTTCCACGGGTCTCTGATCT
RhERFas GCTTCCTAACGCCTCTGTAA

```
CGAAAGTGCCAAGCCAGACTCTCGTCTTCTTCCACGGGTCTCTGATCTCGGCGGCGTAGCGACCCCACGGCCGCTTCC
TAACGCCTCTGTAA
```

Appendix 4. Computer programs used for analysing gene sequences (a) NCBI Blast program, (b) ClustalW program, (c) Chromas and construction of primers (d) Primer 3 Input program.
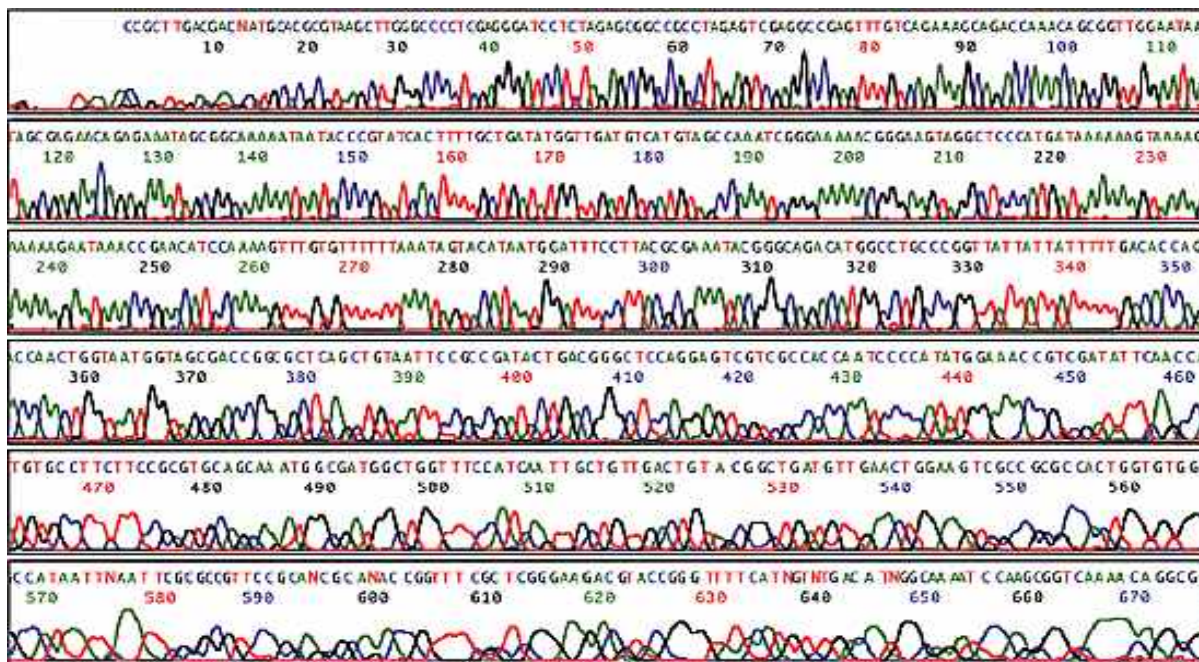
(a). NCBI Blast program

(http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&LAYOUT=TwoWindows&AUTO_FORMAT=Semiauto&ALIGNMENTS=50&ALIGNMENT_VIEW=Pairwise&CLIENT=web&DATABASE=nr&DESCRIPTIONS=100&ENTREZ_QUERY=%28none%29&EXPECT=10&FILTER=L&FORMAT_OBJECT=Alignment&FORMAT_TYPE=HTML&NCBI_G).
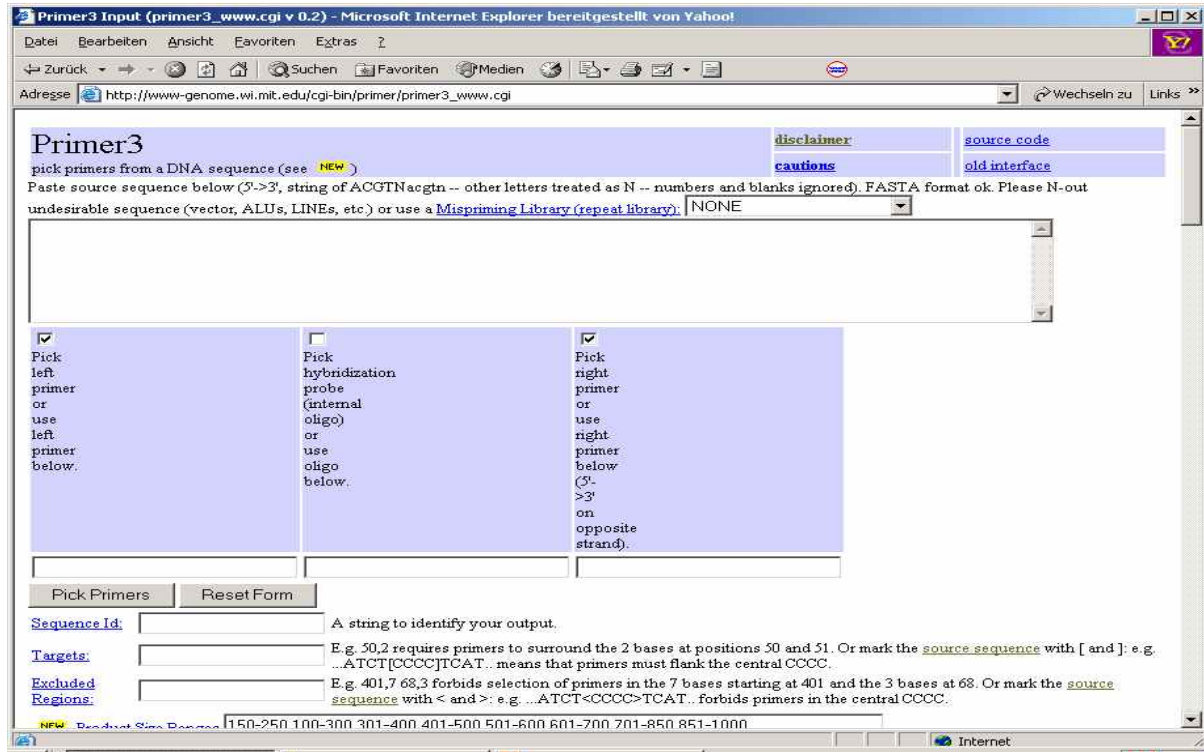
(b). ClustalW program (http://www.ebi.ac.uk/clustalw/).

(c). Chromas (http://www.technelysium.com.au/chromas.html).
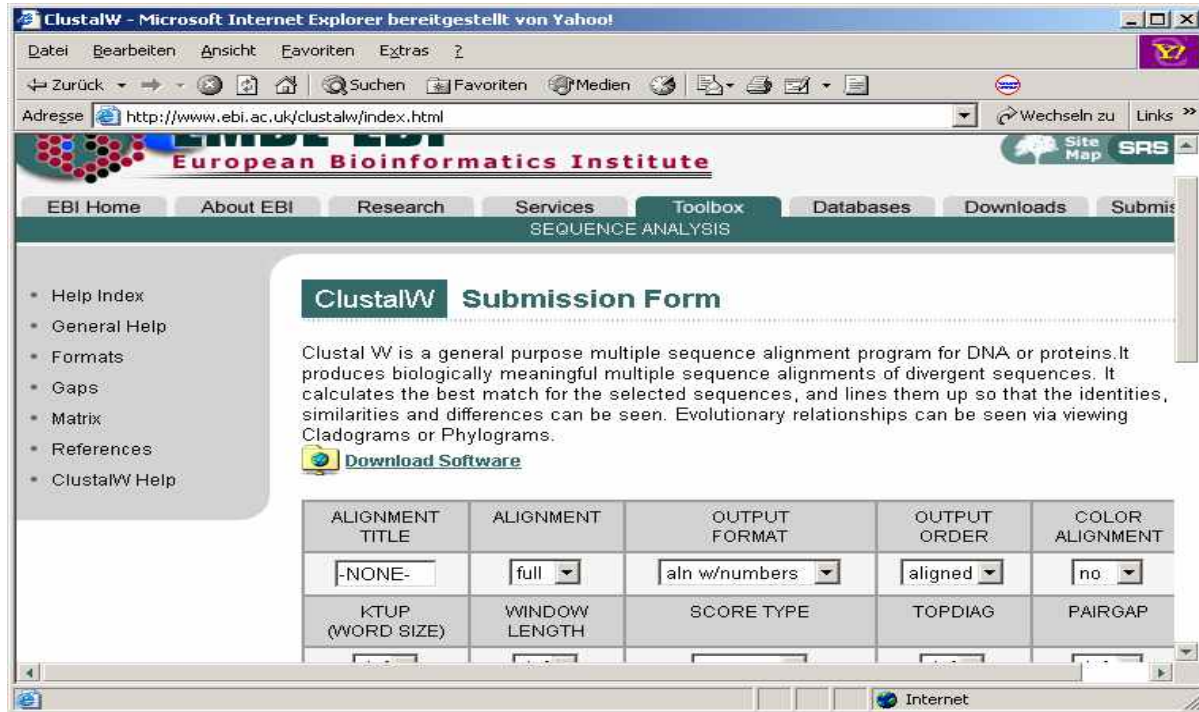
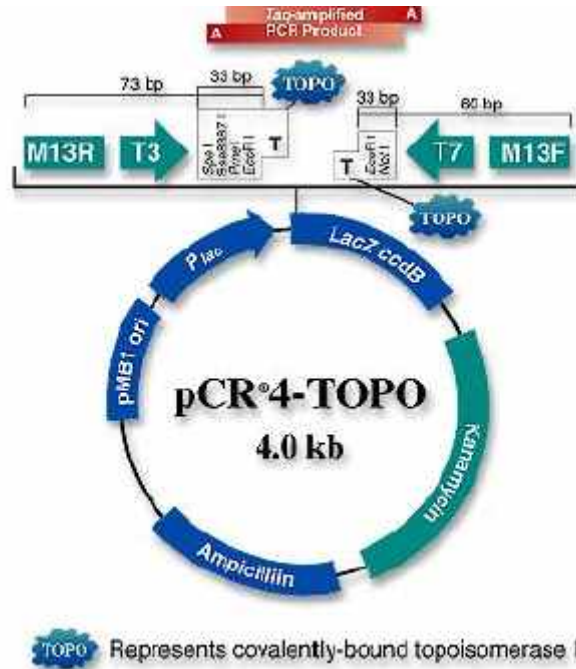(d). Primer 3 Input program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Appendix 5. The principle of ligation into a Topo TA Cloning Kit (Invitrogen, Carlsbad, CA, http://www.invitrogen.com/content/sfs/vectors/pcr4topo_map.pdf).

Appendix 15. The principle of ligation into a Topo TA Cloning Kit (Invitrogen, Carlsbad, CA, http://www.invitrogen.com/content/sfs/vectors/pcr4topo_map.pdf).

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides Statt, das ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe sowie dass diese Arbeit noch nicht als Dissertation oder andere Prüfungsarbeit vorgelegt worden ist.

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