Characterization of Ethylene-Induced Abscission in Miniature Rose (*Rosa hybrida* L.)

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Noorollah Ahmadi, M. Sc. Horticulture

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Referent: Prof. Dr. Margrethe Serek
Korreferent: Prof. Dr. Traud Winkelmann
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Dedicated to my family

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Abbreviations

ABA	abscisic acid
ACC	1-amino cyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylate oxidase
ACS	1-aminocyclopropane-1-carboxylate synthase
AVG	aminoethoxyvinylglycine
bp	base pair
°C	degree Celsius
cDNA	complementary DNA
CTR	constitutive triple response
cv.	cultivar
DDRT-PCR	differential-display RT-PCR
EIL	EIN3-like
EIN	ethylene insensitive
ERF	ethylene response factor
ERS	ethylene response sensor
ETR	ethylene resistant
etr	ethylene resistant mutant
h	hour
IAA	indoleacetic acid
LAC	laccase
1-MCP	1-methylcyclopropene
MAP	mitogen-activated protein
min	minute
mRNA	messenger RNA

NCED	9-cis-epoxycarotenoid dioxygenase
PCD	programmed cell death
PCR	polymerase chain reaction
PDS	phytoene desaturase
PVX	potato virus X
QRT-PCR	quantitative reverse transcriptase real-time PCR
RACE	rapid amplification of cDNA ends
RNA	ribose nucleic acid
RT-PCR	reverse transcriptase PCR
s	second
SAG	senescence-associated gene
SAM	S-adenosyl-methionine
STS	silver thiosulfate
TDZ	thidiazuron
TRV	tobacco rattle virus
VIGS	virus-induced gene silencing

Zusammenfassung

Charakterisierung der ethyleninduzierten Abscission bei Topfrosen (*Rosa hybrida* L.)

Ethylen zeigt bei Topfrosen viele Effekte, die zu einer Verringerung der Nacherntequalität führen: zum Beispiel das Abwerfen von Blättern, die Blütenseneszenz und den Verlust von Knospen und Blüten. Um ethyleninduzierte Gene zu isolieren wurde eine Differential Display PCR bei ethylenbehandelten Topfrosen der Sorte 'Lavender' durchführt. Insgesamt konnten 88 hochregulierte und 72 herunterregulierte Gene detektiert werden. 17 der hochregulierten cDNA Fragmente konnten nach Klonierung und anschließender Sequenzierung verifiziert werden. Sieben cDNA Fragmente zeigten keine Homologie zu bekannten Sequenzen aus Datenbanken und fünf Fragmente zeigten eine Homologie zu Genen aus Bakterien, Pilzen oder Menschen. Von den insgesamt fünf ethyleninduzierten cDNAs wurden drei cDNAs aus Blattstielen und zwei cDNAs aus Blütenstielen isoliert. Zur Analyse der relativen Expression dieser Gene wurde die qRT-PCR eingesetzt. Die relative Expression von allen cDNAs war in den Blütenstielen höher als in den Blattstielen. Die vorliegende Arbeit beschreibt ein ethyleninduziertes, laccasehomologes Gen, das als *RhLAC* bezeichnet wurde. Die komplette cDNA Sequenz des Gens *RhLAC* beträgt 2005 bp und codiert für ein mögliches Protein mit 573 Aminosäuren, die 3 konservierte Sequenzen einer Multi-Kupfer-Oxidase Familien aufweisen. Die Analyse der abgeleiteten Aminosäuresequenz dieses Gens zeigt eine Homologie von 58% zu einer möglichen Laccase aus *Zea mays* (Akzessionsnummer CAJ30499) und eine Homologie von 56% zu der Laccase 15 (Akzessionsnummer NP_100621) aus *Arabidopsis thaliana*. Durch Southern Hybridisierungen konnten mehrere Kopien des Gens *RhLAC* in diploiden und tetraploiden Rosen nachgewiesen werden. Die Expression des Gens *RhLAC* wurde in unterschiedlichen Geweben untersucht. Die höchste relative Expression des Gens *RhLAC* konnte in der Abscissionszone der Blätter nachgewiesen werden, und zwar in dem basalen Bereich des Blattstiels (Pulvinus) (LANZ) und in der Abscissionszone der Knospe, einem kleinen Segment des Blütenstiels (BANZ). Die geringste relative Expression wurde in den Blättern, in den Blattstielen bzw. den Blütenstielen gefunden.

Um die Funktion der Laccase zu charakterisieren wurde die Translation des *RhLAC*-homologen Gens bei *Nicotiana benthamiana* mittels Virus-Induced Gene Silencing (VIGS), durch die Inokulation mit *Agrobacterium tumefaciens*, die mit einem Tabbacco Rattle Virus (TRV) transformiert wurden, unterdrückt. Fünf Tage nach der Infiltration wurden die Pflanzen mit Ethylen behandelt. Sowohl die Unterdrückung der Transkription des laccasehomologen Gens als auch die Ethylenbehandlung reduzierten die Pflanzenhöhe, die gesamte Blattfläche und die Anzahl der Blätter pro Pflanze. Die Ethylenbehandlung beeinflusste nicht die Anzahl der Blätter bei den Pflanzen mit

unterdrückter Laccase-Aktivität. In Pflanzen mit unterdrückten *RhLAC* Gen und bei den Kontrollpflanzen führte die Ethylenbehandlung zur Vergilbung und schließlich zum Absterben der Blätter. Einen ähnlichen Effekt einer erhöhten Anzahl von gelben und abgestorbenen Blättern zeigten auch die Pflanzen mit der gehemmten Laccase.Translation ohne eine Ethylenbehandlung.

Um Genotypen einer Nachkommenschaft, die aus einer reziproken Kreuzung zwischen 'Lavender' und 'Vanilla' entstanden ist, mit einer hohen und niedrigen Ethylensensitivität zu selektieren, wurden 232 Genotypen für 15 Tage mit 1,5 µl/l Ethylen behandelt. Basierend auf dem Merkmal Blattabscission, wurden sieben Genotypen mit der höchsten und 25 Genotypen mit der geringsten Blattabscission für genauere Untersuchungen selektiert.

Der Einfluss der Ethylenbehandlung auf die Blatt- und Knospenabscission wurden für 15 Tage untersucht. Schließlich wurden drei Genotypen mit der geringsten Sensitivität und zwei Genotypen mit der höchsten Sensitivität von der F_1 Nachkommenschaft für physiologische und molekulare Untersuchungen verwendet. In den sensitiven Genotypen war die Chlorophylldegeneration viel höher als in den weniger sensitiven Genotypen. Nach zwei Wochen war die Chlorophylldegeneration bei den weniger sensitiven Genotypen sehr gering. Die Expression der Ethylenrezeptor-Gene (*RhETR1/3*) and der Signal-Transduktions-Gene *(RhCTR1/2*) konnte nicht mit der Ethylensensitivität der untersuchten Genotypen korreliert werden. Die ethyleninduzierte Expression der Laccase (*RhLAC*) war bei den hochsensitiven Genotypen und bei 'Lavender' viel höher als in den Genotypen mit einer geringen Sensitivität und 'Vanilla'. Die geringste Erhöhung der Transkription, die die niedrigste Knospenabscission aufwiesen. Die Induktion der Laccase sowohl als Antwort zum Ethylen, als auch auf biotischen und abiotischen Stress, könnte mit der Pflanzenreaktion auf Stress verbunden sein. Damit ist die Laccase Expression möglicherweise mit dem Prozess der Abscission verbunden der durch biotischen und abiotischen Stress induziert wird.

Schlüsselwörter: Abscission, Differential Display, Ethylen, F1 Hybride, Genexpression, Laccase, Real-Time PCR, *Rosa hybrida*, VIGS

Abstract

Characterization of ethylene-induced abscission in miniature rose (*Rosa hybrida* L.)

Ethylene has deleterious effects on miniature potted roses that leads to loss of postharvest display quality appearing as leaf abscission, flower senescence and bud and flower drop. To isolate ethylene-responsive genes, we used differential display PCR in miniature potted rose 'Lavender' exposed to ethylene. 88 up-regulated and 72 down-regulated genes were detected. Seventeen verified up-regulated cDNA fragments were cloned and sequenced. Seven cDNA fragments did not show any similarity to database and five fragments showed similarity to bacterial, fungal or human genes. Out of five isolated ethylene-responsive cDNAs, three cDNAs were isolated from petiole and two cDNAs from pedicel. QRT-PCR was used to examine the relative expression of studied genes. The relative expression levels of all cDNAs in pedicel were higher than in petioles. This study reports the identification of an ethylene induced homologous laccase, designated as *RhLAC*. The full-length cDNA of *RhLAC* contains 2005 bp and encodes for a putative protein of 573 amino acids containing 3 conserved domains characteristic of the multicopper oxidase family. Analysis of the deduced amino acid sequence of this gene indicated 58% identity to putative laccase in *Zea mays* (accession No. CAJ30499) and 56% to laccase 15 (accession No. NP_199621) in *Arabidopsis thaliana*. Southern blot analysis demonstrated multiple copies of *RhLAC* gene in diploid and tetraploid roses. The expression of *RhLAC* was investigated in different tissues. The highest relative expression of *RhLAC* was found in the leaf abscission zone that included small parts of the petiolar pulvinus (LANZ) and the bud abscission zone with a small segment of pedicel (BANZ). The lowest relative expression occurred in leaf blade, petiole and pedicel respectively.

To characterize the functional role of laccase, Virus-Induced Gene Silencing (VIGS) was applied to suppress the *RhLAC* homologue in *Nicotiana benthamiana* inoculated with strains of transformed *Agrobacterium tumefaciens* carrying tobacco rattle virus (TRV) vectors. The infiltrated plants were exposed to ethylene 5 days after inoculation. Silencing laccase homologue and ethylene decreased the height and total leaf area per plant. Ethylene did not affect the leaves numbers of laccase silenced plants. Ethylene increased leaf yellowing that leads to death of leaves in the plants with suppressed translation of *RhLAC* and in control plants. Similarly, laccase silencing increased the numbers of yellow and dead leaves in non ethylene treated plants.

To screen high and low ethylene sensitive offspring genotypes resulting from a reciprocal crossing between 'Lavender' and 'Vanilla', 232 genotypes were exposed to 1.5 µl/l ethylene for 15 days. Based on leaf abscission, 7 genotypes with highest and 25 genotypes with lowest leaves abscission were selected for detail investigation.

Evaluation of leaf and bud abscission after ethylene treatment was carried out for 15 days. Finally, three genotypes with lowest sensitivity and two genotypes with highest sensitivity were chosen from F1 genotypes for physiological and molecular investigations. Chlorophyll degradation increased in more sensitive genotypes much more than in low sensitive genotypes. After two weeks, chlorophyll degradation increased in low sensitive genotypes. Expression of ethylene receptor genes (*RhETR1*/*3*) and signal transduction genes (*RhCTR1*/*2*) was not correlated with ethylene sensitivity of the investigated genotypes. Ethylene-induced expression of *RhLAC* in high sensitive genotypes and 'Lavender' was increased more strongly than in low sensitive genotypes and 'Vanilla'. The lowest level of *RhLAC* transcript was accumulated in pedicels of genotypes that showed the lowest bud abscission. Inducing laccase in response to ethylene as well as biotic and abiotic stresses could be linked to the mechanism of plant reaction to stress. Laccase expression may be may be related to abscission process caused by abiotic or biotic stresses.

Key words: abscission, differential display, ethylene, F1 hybrid, gene expression, Laccase, real-time PCR, *Rosa hybrida*, VIGS

1. Introduction

Senescence is a series of processes that follows physiological maturity and finally leads to the death of a whole plant, organ, tissue or cell (Watada *et al*., 1984). This deteriorative process moves forward into the programmed cell death (PCD). However, a senescence symptom can be reversible, if it has not gone too far. For instance, the visible symptom of senescence appearing as leaf yellowing is reversible before passing the starting point of PCD (Rogers, 2006; van Doorn and Woltering, 2004). Through the plant development procedure, different plant organs are shed via process termed abscission. Indeed, abscission is a mechanism that helps plant to remove senescing or damaged organs and also to release fruits when they are ripened (Sexton and Roberts, 1982; Bleecker and Patterson, 1997).

Abscission and senescence are affected by environmental and internal factors. These factors manipulate a variety of physiological processes and conditions that sequentially accelerate or delay the abscission process. The environmental factors include temperature, light, water, gases, mineral nutrients and parasitic organisms. Carbohydrate content, respiration efficiency, enzymatic reactions and levels of plant hormones are noted as the most important internal factors (Addicott, 1968; Addicott, 1982). Among the plant hormones, ethylene plays important role in leaf senescence, fruit ripening, leaf and fruit abscission (Abeles, 1992). Although abscisic acid (ABA) is involved in leaf senescence phenomenon via its promotion of senescence, it might induce ethylene biosynthesis and stimulate abscission (Taiz and Zeiger, 2002).

The decades studies on reaction of different plants families to ethylene are consistent with the idea that ethylene is the most important agent in different organs abscission and senescence of *Rosaceae* family. Rose (*Rosa hybrida* L.) is the main ornamental crop cultivated around the world, consumed as garden roses, cut flowers and potted roses. The production of miniature potted roses is going to become popular in many parts of the world. Since 1980s, the potted rose market has been expanded due to releasing new varieties and development of year-round production system. Main centers of miniature potted roses production are Denmark, the Netherlands, the United States and Canada (Pemberton *et al*., 2003). The current annual word production of miniature potted roses is estimated at 75 million pots in Europe and 25 million in the USA/Canada (personal communication Torben Moth, production director, RosaDanica A/S, Odense, Denmark).

The key factor for trade and marketing development of potted roses is postharvest longevity (Serek and Andersen, 1993). Except some postharvest diseases, the main deterioration problems occurring during marketing of potted roses are leaf yellowing, wilting of flowers, bud and leaf abscission. These unfavorable disorders are accelerated by ethylene endogenously produced in plants or exogenously by ethylene-contaminated air (Serek, 1993; Serek *et al*., 1994b; Müller *et al*., 1998, 2001a).

The reaction of potted rose plants to exogenous ethylene varies among different cultivars (Müller *et al*., 1998; Buanong *et al*., 2005; Hassan *et al*., 2004). Also the display quality of plant placed in ethylene-free air is dependent on the cultivar (Müller *et al*., 1998; Serek, 1993). These natural variations in response to ethylene could be useful in breeding program for improving postharvest qualities of potted roses. As a genetic resource, cultivar 'Vanilla' is well known for its long display life as it showed low sensitivity to exogenous ethylene (Müller *et al*., 1998).

In rose breeding program, large numbers of F1 breeding lines are evaluated annually in a selection process. Rose breeding companies such as Kordes' Söhne Rosenschulen (GmbH & Co KG, Sparrishoop, Germany) are dealing with about 50,000 F1 breeding lines. The main efforts of these companies are to pick out F1 hybrid plants with attractive flower color and form (personal communication from Proll, research director, Kordes' Söhne Rosenschulen). The investigation of leaves and floral buds tendency to abscission could be considered in breeding and selection of rose cultivars (Goszczynska and Zieslin, 1993). Because of the high cost and time-consuming, less attention is paid on postharvest behavior of selected genotypes especially in relation to exogenous ethylene.

The aim of this research is to develop a selection strategy by characterization of physiological and molecular aspects related to ethylene sensitivity in F1 hybrid of *Rosa hybrida*. Furthermore, isolation of genes induced by exogenous ethylene treatment and analysis of gene function by virus-induced gene silencing technique can bring new information at molecular level of abscission process.

This thesis contains a collection of papers pertaining to organ abscission in miniature roses and isolation and functional analysis of laccase gene under ethylene treatment. General view of senescence and abscission phenomena and also the statement of the problem are given in introduction part. The contemporary research reports were

reviewed in literature review chapter focusing on physiological and molecular genetic characteristics of plant organs abscission and senescence. The experimental data are presented in one article published in Journal of Plant Growth Regulation, an international journal with referee system, two manuscripts (one already accepted and in printing process in Postharvest Biology and Technology) and two conference and poster papers. At the end, the results of all experimental work are discussed, considering the results of previous studies and possible assumption of presented research.

Literature Review

2. Literature Review

2.1 Senescence processes

Senescence as the final stage of plant growth and development is regulated at the cellular, tissue and organ levels. This process is genetically governed and influenced by environmental factors. The complex action of several internal and external signals may be involved in the induction of senescence. For instance, nitrogen deficiency, light limitation and drought stress will initiate the onset of senescence which may occur in early seed development and reduce plant life period (Thomas and Stoddart, 1980; Buchanan-Wollaston, 1997).

Considering the whole plant, different types of senescence have been explained such as monocarpic, sequential and autumnal. Monocarpic senescence seems to take place as a consequence of reproduction and competition for nutrition resources between younger and elder leaves results to sequential senescence. In deciduous trees, the autumnal senescence may appear as a consequence of decreasing daylength and temperature (Smart, 1994). Senescence lead to the death of the entire monocarpic plants after finishing reproductive development while in polycarpic plants, senescence is limited to parts of flower, fruit and old leaf, and the plant continues to develop (Borochov and Woodson, 1989). Apart from the type of senescence, the control steps of senescence may be similar at the molecular level (Smart, 1994).

At the last step of developmental stage of leaves, senescence is begins with rescue of nutrients by transferring them to younger leaves. Also these rescued nutrients are consumed in developing seeds or stored in special organs for the next growing season (Quirino *et al*., 2000). The senescing leaf operates as a source of nutrients to the whole plant, but at the expense of its own ability to survive (Bleecker and Patterson, 1997).

The senescence process is mediated by physiological and biochemical changes including degradation of proteins, nucleic acids and chlorophyll, disruption of membrane, loss of cellular compartments and climacteric increase in respiration. The most visible symptom of leaf senescence, yellowing, is caused by the degradation of chlorophyll (Fang *et al*., 1998; Borochov and Woodson, 1989). While the leaf becomes elder, its assimilatory capacity decreases, mesophyll tissue commences to lose its

chlorophyll and turn yellow or red. The color changes are both attributed to degradation of chlorophylls compared with carotenoids and the synthesis of anthocyanins and phenolics compounds (Matile, 1992).

Ethylene is considered as a major plant growth regulator of senescence of most plant organs (Abeles, 1992). In petal, ethylene can diminish the membrane integrity by degeneration of some membrane components such as phospholipids, resulting in flower senescence (Borochov *et al*., 1997).

2.2 Abscission

2.2.1 Abscission phenomena

The term of abscission or shedding is applied to the process of separation of cells, tissues, or organs from the remaining plant body. Mostly, the abscission is considered as separation of leaves, flowers, fruits and other organs in higher plants since their shedding is visible. Many other tiny structures can be abscised in lower plant, particularly propagules (Addicott, 1982). Abscission, the last step in senescence process, is a developmentally controlled program of cell separation and cell interruption takes place in the cell walls of the abscission zone (Bleecker and Patterson, 1997). In leaves, the abscission zone is placed at the base of the petiole, the joining point of the petiole to stem. In compound leaves that shed their leaflets, the case of miniature rose plants, abscission zone of the leaflet is located at the attachment point of leaflet to petiole (Addicott, 1982). The abscission region can be recognized by smaller cells that filled more tightly with cytoplasm and being more meristematic than the neighboring tissue of stem and petiole (Fig. 1). Other characteristics of cells in abscission zone could be named as having smaller intercellular spaces, higher deposits of starch and lesser deposition of lignin, no-completely developed tracheary elements (Addicott, 1982; Sexton and Roberts, 1982). The cell wall breakdown and the catabolic activities involved in the abscission process occur in the middle lamellae and the primary cell wall of the abscission zone. The first enzymes that reported to be implicated in wall break down are pectin methyl esterase and endo-ß l:4-glucan 4-glucan hydrolase. Popular name of cellulase was adopted to endo-ß l:4-glucan 4-glucan hydrolase, although it may not be able to cleave crystalline cellulose (Sexton and Roberts, 1982).

Increase in activity of cellulase and cellulase-like enzymes was reported in abscission zone of *Lathyrus odoratus* and bean (Sexton and Roberts, 1982; Sexton *et al*., 1990). The two known gene families involved in abscission are the cellulases (endo1, 4-ß-D glucanase) and polygalacturonases (Sexton *et al*., 1985; Taiz and Zeiger, 2002). In addition to cellulase and pectinase, other enzymes such as chitinases, ß-1,3-glucanase, uronic acid oxidase and peroxidases participate in abscission processes (Sexton *et al*., 1985; Brown, 1997).

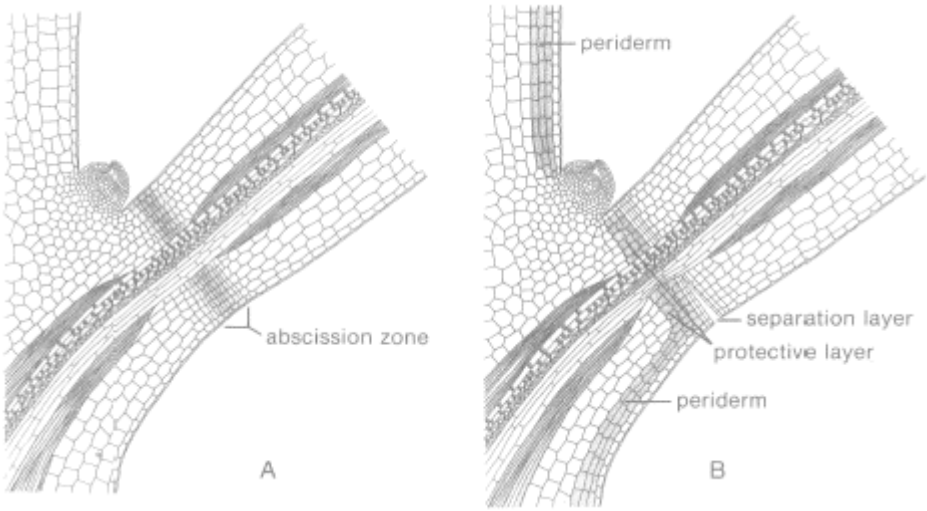

Fig. 1. Diagrammatic view of tissues in a typical leaf abscission zone. A. Before separation. The abscission zone cells are smaller and less differentiated than cells in adjacent regions. B. Compression of vascular tissues by the developing protective layer at time of abscission (Addicott, 1982).

2.2.2 Abscission behavior of different plant organs

2.2.2.1 Leaf and leaflet abscission

Leaf abscission is a natural feature of plant growth and development. As the plant grows, the first developed leaves shed when they become disadvantaged in competition with newly developing foliage (Addicott, 1982). Most of reports support the involvement of ethylene in abscission of leaves as well as other organs, although the abscission in some plants is considered to be non-ethylene mediated. Exogenous

ethylene promotes leaf abscission in a range of plants, but some plants are non- or very weakly affected by ethylene, indicating that ethylene is not the initial factor in leaf abscission (Addicott, 1982; Brown, 1997). Natural leaf abscission is correlated with leaf senescence and it is regulated by some environmental and stress factors. On the other hand, in the most cases abscission and senescence are linked. For these reasons, it is difficult to prove ethylene involvement in natural leaf abscission (Brown, 1997).

In ethylene sensitive plants, ethylene regulates the abscission process in leaves, while auxin suppresses the abscission induced by ethylene. More studies have supported the hint that auxin produced by leaf blade or applied to the cut surface of petiole prevents abscission process in petiole. High amount of auxin transferred from leaf blade to petiole reduces ethylene production and sensitivity of abscission zone to ethylene, and consequently inhibits leaf shedding (Addicott, 1982; Taiz and Zeiger, 2002). According to this idea, the oldest leaves located at the lowest part of plant shed due to decreasing of relative flux of auxin across the abscission zone, as aging occurring (Brown, 1997). In cotton seedlings, the effect of ethylene on abscission was related to leaf position. The still-expanding leaves located on upper node were more sensitive to ethylene than fully expanded leaves on lower part of plant. This differential response to ethylene could be resulted of limited abscission-inhibiting effects of auxin in the abscission zone of the younger leaves (Suttle and Hultstrand, 1991).

Application of ABA on pulvinar tissue distal to abscission zone initiated ethylene production, increased cellulase activity and induced abscission in *Phaseolus vulgaris* (Jackson and Osborne, 1972). The leaf abscission in citrus occurs during re-irrigation after a period of water stress. It was showed that ethylene is a key hormonal factor controlling abscission of leaves and ABA as the initial signal to water stress modulated the level of ethylene biosynthesis in leaves of Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) seedlings grown under water stress (Gómez-Cadenas *et al*., 1996).

Besides the whole leaf, the leaflets also shed from petiole in plants bearing compound leaf. In our study, the rose leaflets were abscised by ethylene treatment. Faba bean breeding lines abscised their leaflets in response to ethylene and inoculation with *Botrytis cinerea* (Hashim *et al*., 1997). Similarly, the leaflets of peanut were defoliated after attack by *Cercospora arachidicola* Hori. The abscission of leaflet could be assigned to increased ethylene production in inoculated plants that takes place after invasion by microorganisms (Ketring and Melouk, 1982). In S*ambucus nigra*, leaflets

abscission is synchronized with the activity increase of ß-1,4-glucanase and polygalacturonase and expansin. Expansins play a role in disrupting hydrogen bonds between cellulose microfibrils and xyloglucans that induce cell wall extension (Taylor *et al*., 1994; Belfield *et al*., 2005).

2.2.2.2 Flower and flower parts abscission

Flower abscission was reported in many families, both in monocotyledons and the eudicotyledons. The period from anthesis to abscission of flowers or flower parts are varying from few hours to many days. Flower parts are never abscised in some species. *Magnolia grandiflora* is a typical plant that abscises stamens and petals about two days after flower opening (Addicott, 1982; van Doorn, 2002). Flower abscission takes place before or after fertilization. Lack of pollination in some species induces the abscission zone and in other species failed fertilization activates the abscission due to competition for carbohydrates (van Doorn, 2002). The abscission zone is usually located at the juncture between the plant body and pedicel. We observed that some floral buds of miniature rose were abscised from above point of pedicel close to the receptacle.

Geranium species among the commercial ornamental crops showed severe problems during shipment and transit, mostly due to high sensitivity of petals. Some genotypes abscise the petals within 1–2.5 h of exposure to very low ethylene concentration. It was shown that fracture takes place at the petal base junction with receptacle by rapid enzymatic cell wall degradation. Microscope observations proved that abscission zone at the petal base consists of small cells containing extensive endoplasmic reticulum and many Golgi bodies and ribosomes (Sexton *et al*., 1983; Evensen *et al*., 1993).

2.2.2.3 Fruit abscission

Although fruit abscission is rarely concerned in floriculture, in pomology most of research has been conducted on management of fruit abscission. Many fruit trees bear more abundance of flowers that set extra fruits, while the trees are unable to support them until ripening stage. The physiological fruitlets abscission is a regulatory mechanism to adjust crop load and alternate bearing. The system of self-regulation may act too strong or unsatisfied. For this reason, prevention of fruitlet abscission or

artificially fruit thinning should be applied on tress bearing an abundance of flowers (Bonghi *et al*., 2000).

Abscission results from cell division activity at the base of the pedicel in abscission zone, just before fruitlets dropping. Some ethylene-releasing compounds like ethephon increased apple fruit abscission rate. Using these compounds resulted in high level of soluble protein and also high activities of polygalacturonase, cellulase and peroxidase enzymes (McCow, 1943; Pandita and Jindal, 1991). Activity of endo-ß-1,4-glucanase during raspberry fruit ripening increased approximately 15-fold and the highest activity was identified in the abscission zone on the surface of receptacle. Application of 1-methylcyclopropene (1-MCP) on green fruits showed that endogenous ethylene induced abscission and increased endo-ß-1,4-glucanase in raspberry (Iannetta *et al*., 2000).

2.2.2.4 Other organs abscission

The abscission of roots, branches, prickles (e.g. in *Rosa* sp.), bark and seeds take place in some plants. Root abscission in *Azolla* seems to be same in physiological and cytological changes as aerial parts (Addicott, 1982). *Wollemia* and *Agathis* are distinguished genera currently recognized in the *Araucariaceae* that abscise easily their branches (Burrows *et al*., 2007). The anatomy of branch abscission is similar to that related to shedding of leaves or flowers. For instance, branch abscission in *Azolla filiculoides* is accompanied by increased activities of cellulase and polygalacturonase and in *Populus tremuloides*, the branch abscission could be prevented by applying auxin (Weaver, 1978; Uheda and Nakamura, 2000).

2.3 Ethylene synthesis and signal transduction pathway

2.3.1 Ethylene synthesis

The biosynthetic pathway of ethylene production has been completely clarified by Yang and Hoffman (1984) and this complex metabolic pathway is known as the Yang cycle. The first step in the ethylene biosynthetic pathway is the conversion of methionine to S-adenosylmethionine (SAM), catalyzed by the enzyme SAM synthase. SAM is converted to 1-amino cyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). ACC is the immediate precursor of ethylene and its oxidation by the enzyme ACC oxidase (ACO) results in the production of ethylene, Co_2 and HCN (Fig. 2).

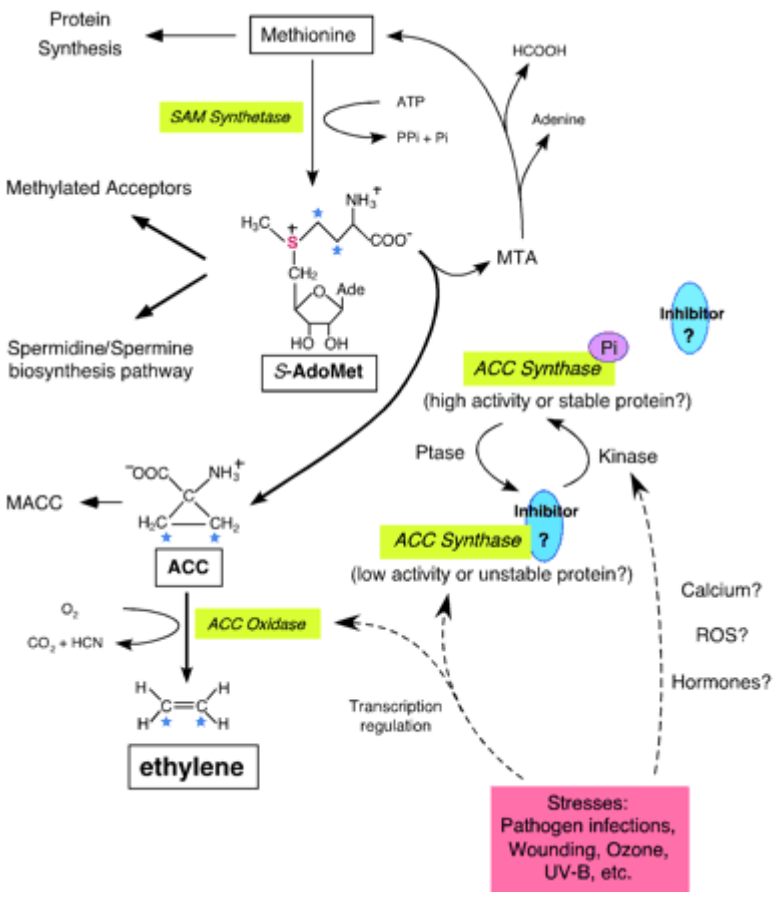

Fig. 2. Biosynthesis pathway and regulation of ethylene by Wang *et al*. (2002).

Ethylene biosynthesis can be regulated by conjugation of ACC. The conjugated form of ACC has been identified as a malonyl conjugate (MACC). Conjugation of ACC may serve as a control of the ethylene production rate (Bufler, 1986). The cloning of *ACS* and *ACO* genes from a number of different species has demonstrated that both enzymes are encoded by multi-gene families. Variety of mechanical and environmental stresses and ethylene regulate the expression of these genes (Kende, 1993).

2.3.2 Ethylene perception and signal transduction pathway

Ethylene is perceived by protein known as ethylene receptor after biosynthesis or absorption of exogenous ethylene. Then ethylene signals transfer via transduction pathway to initiate specific biological responses. The details of biochemical parts of ethylene perception and signal transduction pathway were elucidated by using the model plant *Arabidopsis*. According to the last two decades research, ethylene signaling is negotiated by a family of copper-containing receptors that signal through a pathway. This signaling pathway apparently includes a MAP kinase cascade, a transitional metal transporter, and a transcriptional cascade (Bleecker, 2000; Wang *et al*., 2002).

The first identified receptor, ethylene-resistant (*ETR1*), was cloned by the method of chromosome walking. Four identified *etr1* mutants showed to be dominant to the wild-type allele and the mutation occurred near the amino terminus of deduced protein. The carboxyl-terminal motif seems to have the main characteristic of two-component systems (Chang *et al*., 1993). In general, the system is based on two proteins. The first part, a histidine kinase that acting as sensor, autophosphorylates an internal histidine kinase residue in response to environmental signals. The second fraction is response regulator which stimulates downstream section by receiving a phosphate via aspirate residue from histidine residue of sensor (Bleecker *et al*., 1998; Wang *et al*., 2002).

The second characterized receptor, ethylene response sensor (*ERS*), was isolated by cross-hybridization with the *ETR1* gene. ERS protein showed sequence similarity with the amino-terminal region and putative histidine protein kinase domain of ETR1 (Hua *et al*., 1995). The *ETR2* was cloned and showed sequence homology to the ethylene receptor *ETR1* and *ERS* genes (Sakai *et al*., 1998). *EIN4* and *ERS2* isolated by cross-hybridization with *ETR2* showed higher similarity to *ETR2* than *ETR1* or *ERS1*.

In *Arabidopsis*, the receptor gene family can be divided into two subfamilies based on their structural features. The *ETR1*-like subfamily contains *ETR1* and *ERS1*. This subfamily is distinguished by having three hydrophobic subdomains at the N-terminus and a conserved histidine-kinase domain. The *ETR2*-like subfamily consists of *ETR2*, *EIN4* and *ERS2* receptors genes. This subfamily is characterized by an additional hydrophobic extension at the N terminus. One member of each subfamily missed the C-terminal receiver domain. Fig. 3 shows the structural features of this ethylene receptor gene family (Bleecker *et al*., 1998; Bleecker, 1999; Hua *et al*., 1998). In miniature rose, the ethylene receptors fragments were characterized that showed sequence similarity to the *Arabidopsis thaliana* ethylene receptor gene-family (Müller *et al*., 2000b).

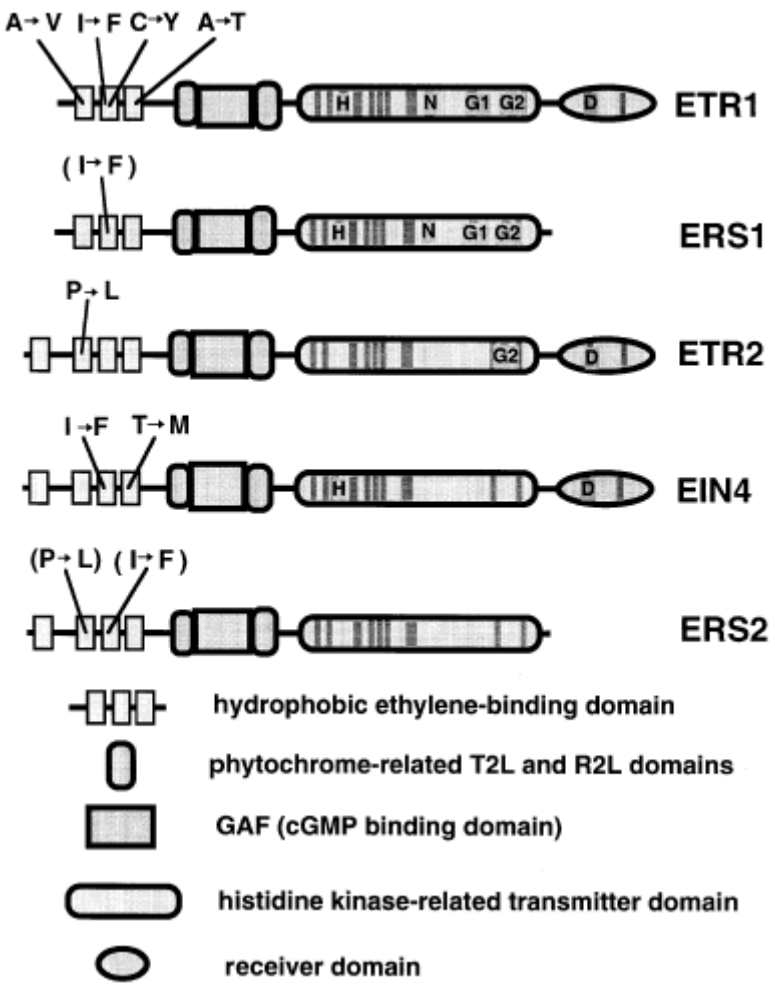

Fig. 3. The structural features of ethylene receptor gene family (Bleecker *et al*., 1998).

Binding of ethylene to receptors results in inactivation of receptors and thereby inactivates downstream components sequentially (Chen *et al*., 2005). Two downstream components identified as *CTR1* and *CTR2* genes are involved in ethylene signal processing. *CTR1* encodes a Raf-like Ser/Thr protein kinase and may be part of a MAP kinase cascade. Genetic analysis places *CTR1* and *CTR2* genes downstream of all five members of ethylene receptors (Hua *et al*., 1998; Bleecker and Kende, 2000). Subsequently, a transcriptional cascade including *EIN3*/*EIL* and *ERF* transcription factors is initiated by *EIN2* activation (Kieber *et al*., 1993; Chen *et al*., 2005). A pathway for ethylene signal transduction represented by Bleecker *et al*. (1998) is shown in Fig. 4.

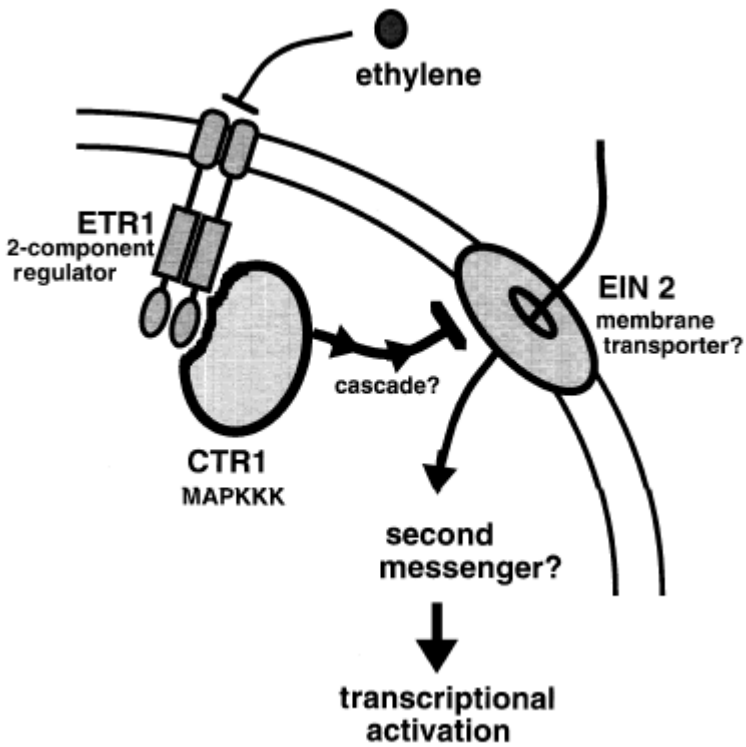

Fig. 4. Model for the transduction of the ethylene signal (Bleecker *et al*., 1998).

2.3.3 Induction and regulation of ethylene biosynthesis and action

Ethylene biosynthesis is well known to be regulated by developmental and environmental factors. Developmental and growth stages like germination and fruit ripening, plant growth regulators, leaves abscission and flower senescence as well as external factors such as environmental stresses persuade ethylene biosynthesis (Abeles, 1992; Liebermann, 1979). Fig. 5 shows some chemicals and molecular genetic inhibition approaches that could be applied to control effect of ethylene (Serek *et al.*, 2006).

Process	Chemical/ environmental inhibition	Molecular genetic inhibition
ACC synthase (ACS)	AVG AOA High CO_2	Antisense ACS Co-suppression ACS
ACC		Heterologous expression of bacterial ACC deaminase
ACC oxidase (ACO)	$CoCl_2$ Alpha-amino*iso*butyric acid Low O_2 concentration High temperature	Antisense ACO Co-suppression ACO
Ethylene gas	Removal by ventilation Chemical removal with $KMnO_4$ Absorption by zeolites	
Receptor	STS 2,5-NBD DACP 1-MCP, CP 3,3, DMCP 3-MCP 1-DCP, 1-HCP	Expressin of mutated ethylene receptor *(etr1-1)*
Responses	Low temperature Cytokinin	Expression IPT gene from *Agrobacterium tumefaciens*

Fig. 5. Mechanisms to control ethylene biosynthesis and action (Serek *et al.*, 2006).

2.3.3.1 Plant growth regulators

Among plant growth regulators, ABA has a strong relationship with ethylene production. In cotton, endogenous ABA had prominent role in ethylene induced cotyledon abscission (Suttle and Hultstrand, 1993). The accumulation of ABA and ACC were increased under water stress in root and xylem flow of Cleopatra mandarin. This result indicates that ABA, the initial sensitive signal to water stress, regulates the levels of ethylene (Gomez-Cadenas *et al*., 1996). In contrast to ABA, application of indoleacetic acid (IAA) or gibberellin (GA3) on decapitated peduncles of' cut flower 'Nubia' delayed the abscission of peduncles (Goszczynska and Zieslin, 1993). Auxin induced ethylene biosynthesis by enhancing *ACS* activity. Previously, some responses mediated by induced ethylene in response to IAA had been ascribed to auxin (Taiz and Zeiger, 2002). Gibberellins are commercially used in monocotyledonous cut flowers such as lilies and *Alstroemeria* to prevent postharvest leaf yellowing (Serek and Reid, 1997). GA_3 retarded chlorophyll degradation and delayed senescence of lettuce leaves, probably by affecting endogenous ethylene production (Aharoni, 1989). Cytokinins, zeatin and their derivatives and analogs prevent leaf yellowing and subsequently leaf senescence. Applying 6- benzyladenine accompanied by silver thiosulfate (STS) reduced leaf yellowing in cut chrysanthemums (Serek and Reid, 1997). The onset of leaf senescence in *Pelargonium* cuttings was significantly delayed by applied thidiazuron (TDZ), recently recognized as cytokinin (Torelli *et al*., 2006). Although cytokinins decrease senescence because of involving in chlorophyll biosynthesis, but it is suggested that the balance between ethylene and cytokinins regulates senescence process (Mutui *et al*., 2005).

2.3.3.2 Environmental stresses

Ethylene biosynthesis is induced by stress conditions such as chilling, drought, flooding, exposure to ozone and mechanical wounding. However, in all these stress conditions, ethylene is synthesized via usual biosynthesis route and the increased ethylene production has been resulted from increase in transcription of *ACS* gene by stress (Taiz and Zeiger, 2002). Miniature rose cv. 'Orange Meillandina' placed in dark condition at 22 °C abscised all young buds after 6 days (Goszczynska and Zieslin, 1993). The simulated transportation stress decreased the postharvest display of potted

miniature rose cultivars 'Bronze' and 'Vanilla' (Müller *et al*., 2000c). Similarly, the flower abscission of *Chamelaucium uncinatum* was caused under stress condition induced by simulated aeroplane transport (Macnish *et al*., 2004).

2.3.3.3 Climacteric ethylene

Senescence together with transient in ethylene production and respiration rate is the distinctive character of climacteric or ethylene sensitive flowers and fruits during senescence or ripening. Carnation, a model ornamental plant, has been well known for its climacteric rise of endogenous ethylene production as well as apple and pear in fruit crops (Woodson and Lawton, 1988; Knee and Looney, 1983). In carnation and gypsophila, the presence of ethylene in the ambient atmosphere or biosynthesized in the plant tissue causes a positive feed-back and subsequently stimulates ethylene biosynthesis (Woodson and Lawton, 1988; van Altvorst and Bovy, 1995). This climacteric rise in ethylene production during senescence of carnation flowers changes the membrane lipids that lead to loss of membrane function. The lipid microviscosity of microtonal membrane increased with climacteric ethylene production. Treatment of flowers with aminoethoxyvinylglycine (AVG) postponed the increasing of membrane microviscosity (Thompson *et al*., 1982). Produced ethylene by gynoecium of senescing carnation flowers acts as signal to provoke the expression of *ACS* and *ACO* in the petals resulted in senescence progress. Apparently, the low ethylene production in the genoecium of cv. White Candle causes low accumulation of *ACS* transcripts leads to its long vase life (Satoh *et al*., 2005). In *Rosa hybrida*, the senescence of flower in some cultivars was accompanied by a rise of ethylene production, while in some cultivars the ethylene production was very low (Müller *et al*., 1998; Ma *et al*., 2006). In *Rosa hybrida* 'Bronze', the increase of ethylene production probably resulted of up-regulation of *ACS* and *ACO* expression (Müller *et al*., 2001c).

2.3.3.4 Pollination-induced senescence

In many species, pollination is an important signal for starting floral cell death and its effects are mediated by ethylene. Other plant growth regulators such as cytokinin and brassinosteroids play role but their role is understood only in some specific systems

(Rogers, 2006). The pollination process promotes ethylene synthesis and also induces premature wilting or abscission of the corolla. In flowers of carnation (*Dianthus caryophyllus* L), senescence is normally associated with production of ethylene, in-rolling of petals and growth of gynoecium (Nichols, 1966, 1971; O'Neill, 1997). Pollen and pistil communication provokes corolla senescence and ovary development in carnation cv. White Sim. It seems that the increase of ethylene production in style is the initial post-pollination response which is followed by ethylene production from the other floral organs (Jones and Woodson, 1997). A dramatic increase in ethylene production occurred by self-pollination in geranium florets leads to floret abscission within 4 h. Transcripts levels of *ACS* and *ACO* were increased in response to self-pollination (O'Neill *et al*., 1993; Clark *et al*., 1997).

In *Pelargonium*, production of ethylene by pollination before fertilization and also effect of exogenous ethylene treatment in senescence and abscission of corolla may be assigned that ethylene could be related to the communication between pollinated stigma and the corolla (Clark *et al*., 1997, Dervinis *et al*., 2000). The senescence induced by pollination was shown differently in different flowers. For instance, in *Lantana camara* flowers, pollination induced anthocyanin synthesis that resulted in petal color change (Mohan Ram and Mathur, 1984). Pollination of carnation and petunia caused flower senescence, wilting and fading (Halevy, 1986).

Pollination in some species reduces drastically the display quality of flowers. Flowers of orchid last several months without pollination and the senescence begins quickly after pollination. In tobacco, senescence is promoted by ethylene evolved after contact between pollens and stigma (O'Neill *et al*., 1993; O'Neill, 1997). The display quality of cut flower of *Phalaenopsis* (*Phalaenopsis* hybrid, cv. Herbet Hager) lasts 2 to 3 weeks. This life span is shorted after pollination, by rapid acceleration of wilting progression. Senescence was induced in *Doritaenopsis*, *Dendrobium* and *Phalaenopsis* by successful pollination (Porat *et al.*, 1994). Pollination often accelerates ethylene biosynthesis and this ethylene is involved in post pollination developmental processes, including petal senescence in tomato flowers (Llop-Tous *et al.*, 2000). Based on these pieces of evidence, it has been suggested that the events of senescence may be orchestrated by the gynoecium enlarging during senescence (Nichols and Ho, 1975). Simultaneousness in the petal senescence and ovary enlargement probably relates to competition between

two organs for taking carbohydrate (Mor *et al.*, 1980). In contrast to pollination, crushing stigma or removing stigma and pistil have no effects on the senescence of *Campanula medium* flowers (Kato *et al.*, 2002).

2.4 Ethylene, senescence and abscission

Patterson and Bleecker (2004) showed that there are two ethylene-dependent and ethylene-independent processes involved in floral organ abscission in *Arabidopsis*. In their studies, ethylene-insensitive mutants of *Arabidopsis* did not need ethylene for activation of abscission processes but abscission occurred in these mutants, whereas the delayed floral organ abscission mutants showed rapid induction of organ abscission in response to applied ethylene.

Ethylene has a regulatory function/effect at transcriptional or/and translational level of genes involved in cell separation (Brown, 1997). The promoter of genes correlated with cell wall degradation is regulated by ethylene during fruit ripening, senescence and leaf abscission (Bleecker and Patterson, 1997). The main genes associated in abscission are from cellulase and polygalacturonases families (Sexton *et al*., 1985; Brown 1997). The inducing role of ethylene in fruit ripening and organs abscission by increasing hydrolytic enzymes activity was previously reported in the *Rosaceae* family (Bonghi *et al*., 1992; Iannetta *et al*., 1999).

2.4.1 Ethylene and senescence

Inducing ethylene synthesis during senescence and delaying senescence by ethylene biosynthesis inhibitors point out the important role of ethylene in this process (Hanley *et al*., 1989; Woodson and Lawton, 1988). Study on *Arabidopsis* suggested that ethylene is not the initial factor to regulate onset of leaf senescence and the ethylene-induced senescence depends on the defined age of individual leaves (Jing *et al*., 2005). Ethylene regulates the petal senescence in some species such as *Petunia* (Xu and Hanson, 2000) and *Campanula medium* (Kato *et al*., 2002).

The distinctive visible symptom of leaf senescence appears as leaf yellowing that caused by breakdown of chlorophyll, compared to other pigments and synthesis of anthocyanins and phenolics compounds (Oh *et al*., 1996). Ethylene accelerates chlorophyll degradation which leading to the enhancement of tissue yellowing in plant leaves (Able *et al*., 2002, 2003). Chlorophyll degradation was delayed in broccoli by 1-MCP while it was accelerated by exposure to ethylene, especially for chlorophyll A (Gong and Matheis, 2003). Pre-storage treatment with 1-MCP delayed yellowing of

broccoli florets and rocket (*Eruca sativa* Mill.) leaves and increased leaves shelf life (Gong and Matheis, 2003; Koukounaras *et al*., 2006). Alejar *et al*. (1988) showed that the rate of ethylene production of leaves from fast yellowing cultivars of tobacco was markedly higher than in the slow yellowing ones. Chlorophyll is broken down into simpler compounds like the phytol, Mg+, and an initial cleavage product of porphyrin moiety. Ethylene increased the chlorophyllase that cleaves chlorophyll into phytol and chlide, the Mg- porphyrin moiety of chlorophyll (Matile *et al*., 1996; Rodoni *et al*., 1997).

The stressful conditions and hormones stimulate senescence and hasten yellowing process related to ethylene production. For instance, drought, darkness, detachment of leaves, and plant hormones like ABA and ethylene can induce visible yellowing (Oh *et al*., 1996; Mutui *et al*., 2005; Becker and Apel, 1993). Detached leaves of barley incubated in darkness showed rapidly increase of the senescence symptoms as indicated by damaged chlorophyll (Becker and Apel, 1993). The expression of several senescence-associated genes (SAGs) in response to age, dehydration, darkness, abscisic acid, cytokinins, and ethylene treatments was studied in *Arabidopsis thaliana* leaves by Weaver *et al.* (1998). Most of the SAGs responded positively to the mentioned treatments in same way while the ethylene and darkness treatment on detachment leaves showed higher induction on SAGs and visible yellowing. Dark-induced expression of senescence-associated gene (*sen1*) was not blocked by cytokinin or $CaCl_2$ that delayed loss of chlorophyll (Oh *et al*., 1996). The cytokinins are well known as senescence retarding hormones, especially in leaves (Borochov and Woodson, 1989). TDZ-treated leaves of *Pelargonium* had high chlorophyll contents, while it was declined in the untreated controls (Mutui *et al*., 2005).

2.4.2 Ethylene and abscission

Ethylene is well documented in induction of fruit abscission in *Rosaceae*. In apple and peach, the abscission was increased with evaluation of *ACO* induced the ethylene production and concomitantly increasing of polygalacturonasese (Bonghi, 1992; Dal Cin *et al*., 2005).

In the petal abscission zone of *Rosa bourboniana* expression of different ethylene-induced genes was characterized. These genes could be noted as expansin gene, PCD gene, a pleiotropic drug resistance like gene, a plastid *FtsH* protease like gene, and a cysteine protease (Sane *et al*., 2007; Personal Communications with Tripathi, Plant Gene Expression Lab, National Botanical Research Institute, Lucknow, India). Recently, a correlation between expansin activity and leaflet abscission induced by ethylene was demonstrated in *Sambucus nigra* (Belfield *et al*., 2005).

Flower abscission and expression of *RhETR2* and *RhETR3* were increased in rose by ABA. These effects of ABA could be result from modulation in ethylene sensitivity (Serek and Reid, 1997; Müller *et al*., 2001b). Two ethylene receptors were isolated from developing and abscising peach fruit termed *PpETR1* and *PpERS1*. The transcription level of *PpETR1* did not show any change but *ERS1* mRNA abundance increased in ripening mesocarp, leaf and abscission-zone of fruitlets. Increasing in ethylene synthesis occurring with accumulation of *PpERS1* indicated that the *PpERS1* might play a role in peach abscission and ripening (Rasori *et al*., 2002). Occurring dominant mutation in receptors loci disarms the plant to be responsive to ethylene, while the wild-type plant reacts well (Bleeker *et al*., 1988). Using analogues to 1-MCP such as 1-decylcyclopropene (1-DCP) and 1-octylcyclopropene (1-OCP) could inhibit ethylene action to improve the display quality of flowers. Compare to 1-MCP, the efficiency of 1-DCP and 1-OCP in protection of ethylene-induced organs of miniature rose cv. 'Lavender' was lower (Buanong *et al*., 2005).

Some cellulase genes were isolated from flower abscission zone of tomato (de1 Campillo and Bennett, 1996) and bean (Tucker *et al*., 1988). The mRNA levels of these genes were accumulated in the presence of ethylene. Some studies showed that contribution of different genes induced the flower and fruit abscission in tomato. Expression of endo-ß-1,4-glucanase (EGase) in abscising flower and ripening tomato fruit showed the overlapping activity of different gene transcripts including EGase1 and EGase2. The imperfect abscission of Cel1transgenic flowers indicated that the Cel1 activity is not sufficient to induce floral abscission in tomato (Lashbrook *et al*., 1998).

2.5 Non ethylene related senescence

Ethylene has no role in flower senescence in some ornamental plants, especially in most monocotyledonous species. Although ethylene-sensitive wilting occurred in the *Alismataceae* and *Commelinaceae,* but no flower abscission was observed (van Doorn, 2001 and 2002). Petals of the dicotyledonous species are generally sensitive to ethylene, except for some families such as *Crassulaceae*, *Gentianaceae* and *Fumariaceae*, and one subfamily in both the *Ericaceae* and *Saxifragaceae* (Woltering and van Doorn1988; van Doorn, 2001; Han and Miller, 2003).

Woltering and van Doorn (1988) classified the senescence and abscission behaviors of more than 90 species belong to 22 families. According to their studies, generally the species of the *Compositae* and *Iridaceae* families and most of the *Amaryllidaceae* and *Liliaceae* species were evaluated as low sensitive plants to ethylene treatment. In another investigation, *Cymbidium* hybrid species also showed to be insensitive to ethylene, while other monocotyledon species such as *Yucca intermedia*, *Y. filamentosa, Agapanthus africanus*, *Kniphofia uvaria* and *Asparagus* sp. showed high ethylene sensitivity on flower abscission (van Doorn, 2002). *Tulipa* did not show any ethylene climacteric pattern and the ethylene production remained at low level. It was deduced that ethylene does not have primary regulation effect on tepal falling (Sexton *et al.*, 2000).

Applied ethylene had no influence on senescence of the florets of the *Gladious* cultivars (Serek *et al*., 1994a). No effect of exogenous ethylene was showed by oriental lily. In this plant, ethylene-exposed fresh flowers, buds and entire cut stem did not show undesirable effects on bud opening or longevity or the development of leaf yellowing (Han and Miller, 2003). The response of *Lilium* to ethylene exposure is often contradictory and it seems to be related on species and cultivars, or even the storage conditions. For instance, treatment of the Asiatic hybrid lilies with ethephone increased the percentage of flower abscission and bud abortion (Song and Peng, 2004). In contrast, vase life of most oriental hybrid and *Lilium longiflorum* cultivars was unaffected by ethylene exposure (Elgar *et al.*, 1999). The reaction of *Lilium* may depend on species and cultivars. Two *Lilium* cultivars, Connecticut King and Enchantment, reacted differently to inhibitors of ethylene biosynthesis such as AVG and cobalt

chloride. Both ethylene inhibitors prevented flower abscission of the cv. Enchantment, without any effect on the cv. Connecticut King (Malorgio *et al*., 1996).

It was shown that sensitivity of lily 'Stargazer' buds increased during cold storage, while the buds were insensitive without cold storage treatment (Han and Miller, 2003). The assumption of increasing sensitivity to ethylene is based on the developing and maturing buds during storage (Whitehead *et al*., 1984), or modulation of receptor levels during development or cold storage induced stress (Payton *et al*., 1996). Moreover, produced endogenous ethylene was revealed following cold storage, while it was not detected in freshly harvested buds and leaves (Han and Miller, 2003). Ethylene production was also stimulated in the youngest buds of *Gladiolus* by cool storage (Serek *et al*., 1994a). Although small amount of ethylene was produced immediately prior to abscission but the senescence process in *Alstromeria* is independent of ethylene production (Wagstaff *et al*., 2005). Ethylene seems to play little or no direct role in senescence of some species of *Hemerocallis* and *Alstroemeria* (Woltering and van Doorn, 1988; Wagstaff *et al*., 2005).

Publications

3. Publication

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Isolation of an Ethylene-induced Putative Nucleotide Laccase in Miniature Roses (*Rosa hybrida* L.)

Noorollah Ahmadi · Heiko Mibus · Margrethe Serek

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Abstract Using differential display we isolated five ethylene-responsive cDNAs from *Rosa hybrida* L. and identified for the first time an ethylene-induced cDNA homologous to a laccase gene. Three cDNAs were isolated from petioles and two cDNAs from pedicels. Expression levels of all cDNAs in pedicels were higher than in petioles. The laccase homolog cDNA was termed the *RhLAC* (*Rosa hybrida* Laccase) gene. The *RhLAC* gene encodes for a putative protein of 573 amino acids containing three conserved domains characteristic of the multicopper oxidase family. Southern blot hybridization analyses indicated that there are multiple copies of the *RhLAC* gene in the *Rosa* species. Comparison of the relative expression of isolated *RhLAC* in various organs showed that it was highly induced in the leaf abscission zone of petioles and the bud abscission zone of floral bud pedicels, whereas it was low in both leaf blades and petioles. These results suggest that *RhLAC* may play an important role in senescence and abscission in roses.

Keywords Ethylene · *Rosa hybrida* · Laccase · Real-time PCR · Differential display

Introduction

Ethylene is a gaseous hydrocarbon plant growth regulator; at trace amounts it has a major influence on plant growth and development, including the processes of fruit ripening; leaf, floral bud, and flower senescence; and abscission. Commercially, abscission of leaves or floral organs reduces marketability of horticultural products such as cut flowers and potted plants (Sisler and Yang 1984; Reid 1995).

The repertoire of physiologic effects caused by ethylene is extensive, but the main effects of endogenous and exogenous ethylene on potted rose plants include hastening of leaf and bud abscission, premature flower senescence, reduced flower longevity, enhanced petal abscission, and leaf yellowing (Serek and Reid 1994; Müller and others 1998, 2001a), although ethylene is not the initial factor in leaf yellowing (Serek and others 1996). The behavior of potted roses varies in response to exogenous ethylene treatment depending on cultivar (Müller and others 1998; Hassan and others 2004; Buanong and others 2005). Studies on 50 plant families from monocotyledons and eudicotyledones showed that flower abscission was highly related to ethylene in all species except *Cymbidium* (Van Doorn 2002). In the cut rose (*Rosa hybrida*) cv. Samantha, exogenous ethylene induced endogenous ethylene biosynthesis in petals (Ma and others 2006).

In roses, as in *Arabidopsis,* ethylene is perceived by a family of receptors that function similarly to the bacterial two-component histidine kinases (Bleecker and Kende 2000; Müller and others 2000; Wang and others 2002). Ethylene treatment increases transcript levels of some receptors and of constitutive triple-response genes (*CTRs*) in the cut rose cv. Samantha and *Delphinium* (Kuroda and others 2004; Ma and others 2006).

Abscission processes result from catabolic activities in the middle lamellae and the primary cell wall of the abscission zone. The two main gene families involved in abscission are the cellulases (endo1, 4-β-D glucanase) and polygalacturonases (Sexton and others 1985; Brown 1997).

N. Ahmadi · H. Mibus · M. Serek (✉)
Faculty of Natural Sciences, Institute for Ornamental and Woody Plant Science, Leibniz University of Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany
e-mail: Serek@zier.uni-hannover.de

H. Mibus
e-mail: mibus@zier.uni-hannover.de

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Although ethylene has a regulatory function/effect at the transcriptional or/and the translational level, expressed genes are also involved in cell separation (Brown 1997), but it is not clear if ethylene is the specific inducer of cell separation or whether it promotes the abscission process per se (Gonzalez-Carranza and others 1998). Patterson and Bleecker (2004) believe that both ethylene-dependent and ethylene-independent processes are involved in floral organ abscission. In their studies, ethylene-insensitive mutants of *Arabidopsis* did not need ethylene for activation of abscission processes but abscission occurred in these mutants, whereas the delayed floral organ abscission mutants (*dab*) showed rapid induction of organ abscission in response to applied ethylene.

It was shown that branch abscission of *Azolla filiculoides* induced by ethylene was associated with increased activities of cellulase and polygalacturonase (Uheda and Nakamura 2000). Some cellulase genes were isolated from the flower abscission zone of tomato (del Campillo and Bennett 1996) and bean (Tucker and others 1988) and mRNA levels accumulated in the presence of ethylene. In addition to cellulase and pectinase, other enzymes, including chitinases and β-1,3-glucanase, uronic acid oxidase, and peroxidases, have been associated with abscission processes (Sexton and others 1985; Brown 1997). After exposure to ethylene, some genes, including a programmed-cell-death-like gene, a pleiotropic drug-resistance-like gene, a plastid *FtsH* protease-like gene, and a cysteine protease, were expressed in the petal abscission zone of rose (personal communications with Tripathi, 7th International Symposium on the Plant Hormone Ethylene, Pisa, Italy, 2006).

Recently, a correlation between expansin activity and leaflet abscission induced by ethylene was demonstrated in *Sambucus nigra* (Belfield and others 2005). In the petal abscission zone of *Rosa bourboniana*, expression of the expansin gene was upregulated by ethylene, and it was also induced under natural conditions (Sane and others 2007). Expansins play a role in disrupting hydrogen bonds between cellulose microfibrils and xyloglucans that induce cell wall extension (Belfield and others 2005). Briefly, all genes encoding proteins associated with cell-wall disassembly during fruit ripening, leaf abscission, and leaf senescence contain promoter elements regulated by ethylene (Bleecker and Patterson 1997).

To identify new genes, it is necessary to isolate differentially expressed genes in various kinds of cells or under different conditions. Differential display RT-PCR was invented to simplify and accelerate identification of differentially expressed genes (Liang and Pardee 1992; Liang 2002). Differentially regulated ethylene-responsive genes were isolated during abscission of peach fruitlets using this method (Ramina and others 1999).

We attempted to isolate novel genes induced by exogenous ethylene in leaf petioles and bud pedicels of the ethylene-sensitive *Rosa hybida* L. cv. Lavender. We used the differential display RT-PCR technique and identified five ethylene-responsive cDNAs, including a laccase cDNA.

Materials and Methods

Plant Materials and Growth Conditions

Miniature rose plants (*Rosa hybrida* L. cv. Lavender) from the Kordana breeding line (W. Kordes' Söhne Rosenschulen GmbH & Co KG, Germany) and a diploid rose genotype 94/1 (Debener and Mattiesch 1999) were used in this experiment. Plants were propagated from cuttings in the experimental greenhouse at Leibniz University of Hannover, grown three cuttings per pot at a temperature of 20°C/20°C (day/night) and 70% relative humidity (RH). Day length was extended to 16 h by SON-T lamps (Osram, 400 W, Philips Co.), which supplied 260 μmol m^{-2} s^{-1}.

Exogenous Ethylene Treatment

Potted miniature rose plants cv. Lavender were placed in 200-L glass chambers. The chambers were ventilated with air carrying ethylene of 1.5 (±0.25) μl l^{-1} (Saltveit 1978). Control plants were placed in identical glass chambers under the same conditions but without ethylene. Ethylene concentration was monitored daily using a Perkin-Elmer portable digital gas chromatograph (GC Voyager FFKG312) equipped with a photoionization detector. Oven and column temperatures were set on 60°C, injection pressure was 69 kPa, and the N_2 carrier gas flow was 40 ml min^{-1}.

After 24, 48, and 72 h exposure to ethylene, bud pedicel and leaf petiole explants were harvested. In addition, leaf abscission zones (LANZ), bud abscission zones, (BANZ), leaf blades, and petal samples were collected 72 h after ethylene treatment. The LANZ sample consisted of a stem-petiole abscission zone and 2 mm of basal petiole attached to the stem containing a stipule. The BANZ sample consisted of a floral bud abscission zone with 2 mm of the basal part of the floral bud pedicel. Ethylene treatment was repeated three times and samples were collected from 80 potted plants at this sampling time.

Plant samples were frozen immediately in liquid nitrogen, ground with a mortar and pestle in liquid nitrogen, and then stored at −80°C until extraction of RNA.

Extraction of RNA

Total RNA was isolated from 40–60 mg of ground plant samples using the Invisorb® Spin Plant RNA Mini Kit

(Invitek Co.) according to the manufacturer's protocols. Concentration of RNA was determined by measuring absorbance at 260 nm (SmartSpec 3000 Spectrophotometer; Bio-Rad). To evaluate RNA quality, total RNA was fractionated on 1% agarose gel visualized by staining with ethidium bromide and compared with standard concentrations of λDNA (Fermentas Co.). For removing genomic DNA, RNA samples were treated with DNase I (Fermentas Co.) as follows: 0.5 U of DNase I, 1 µl of 10 × DNase reaction buffer (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.6), and 20 U of RiboLock™ (Fermentas Co.) were added to each 2-µg RNA sample and incubated in a thermocycler (Biometra Co.) at 37°C for 30 min. To eliminate residues of DNase enzyme, 40 µM EDTA was added to each RNA sample followed by incubation at 65°C for 10 min. To avoid any degradation, RNA samples were stored at −20°C for the short term or −80°C for long term until further use.

Reverse Transcription and Differential Display PCR

Purified total RNA (0.5 µg) extracted from 72-h ethylene-treated bud pedicels and leaf petioles and from control samples was reverse-transcribed using M-MLV RT, RNase H Minus: a point Mutant (Promega Corp.). Reverse transcription was carried out using 2.5 µM anchor primer (D1-D12, Biometra Co.), incubation at 70°C for 7 min, and quenching immediately on ice for 5 min. After a brief centrifugation, 2.5 µl of M-MLV RT 5 × buffer, 0.5 mM dNTPs, 200 U of M-MLV RT (H-) enzyme, and 20 U of RiboLock™ (Fermentas Co.) were added to the RT reaction and incubated at 40°C for the first 10 min, 50°C for 150 min, and finally 70°C for 5 min. PCR amplification was carried out in a 20-µl reaction mixture containing 10 ng of cDNA template, 150 µM each of dNTP, 2.5 µM downstream primer (D1-D12), 0.5 µM upstream random primer (U1-U26), 0.5 U Taq DNA polymerase (Invitek Co.), 10 mM Tris-HCl, 50 mM KCl and 2 mM MgCl. PCR amplification was conducted in the thermocycler (Biometra Co.) under the following conditions: 30 s at 94°C for initial denaturation, followed by 46 cycles consisting of 30 s at 94°C for denaturation, 1 min at 40°C for annealing, 1.5 min at 72°C for polymerase extension, and a final extension step of 72°C for 10 min.

PCR reaction products were separated by 1% agarose gel stained with ethidium bromide and the sizes of amplicons were evaluated by comparing them to a 100-bp DNA marker (Fermentase Co.). The amplified polymorphic bands that occurred as a result of ethylene treatment were cut from the gel and stored at 4°C. For cloning, the DNA fragments were purified from agarose gels using the Invisorb® Fragment CleanUp kit (Invitek Co.).

Cloning, Sequencing, and Sequence Analysis

The upregulated fragments were ligated into TA plasmid vector using the pCR®4-TOPO® TA Kit (Invitrogen) according to manufacturer's instructions. Positive-transformed bacteria were selected by PCR reaction using 24 ng of T3 and T7 primers and targeted clones were incubated in 5 ml of liquid LB medium containing 150 µg ml^{-1} ampicillin overnight at 37°C.

Plasmid DNA from transformed *E. coli* was isolated using the NucleoSpinR Plasmid Kit (Macherey-Nagel Co.) and sequenced by MWG Biotech Co. The isolated sequences were analyzed using the CLUSTAL W program, European Bioinformatics Institute (EMBL) (Higgins 1994) and the homology search was done using the BLUSTN program (National Center for Biotechnology Information, NCBI) (Altschul and others 1997).

To validate results, specific primers were designed using the Primer3 program (Rozen and Skaletsky 2000). The annealing temperature of commercially synthesized specific primers was optimized using the Primus 96 advanced® Gradient thermocycler (Peqlab Co.).

Real-Time RT-PCR Assay

To quantify mRNA levels, real-time RT-PCR assays were performed using the Rotor Gene 3000 real-time thermal cycler (Corbett Life Science Co.). Reverse-transcription reactions were performed using 1 µg of oligo-dT(23) primer as described previously in the Reverse Transcription and Differential Display PCR subsection. The PCR reaction mixture was made up to a volume of 20 µl containing 10 ng of cDNA template, 150 µM of each dNTP, 0.25 µM forward primer, 0.25 µM reverse primer (Table 1), 1 U of Hot Start Taq DNA polymerase (Axon Co.), 5 mM $MgCl_2$, 10 mM Tris-HCl and 50 mM KCl. Amplification of PCR products was monitored via intercalation of SYBR® Green (Roche Applied Science Co.) added to the reaction mixture in each tube. After 10 min of incubation at 94°C, the cDNA was amplified by 45 three-step cycles: 30 s at 94°C, 1.5 min at 62°C, and 2 min at 72°C. To normalize all samples, the expression levels of β-actin, a housekeeping gene, were detected for each sample concomitantly with specific genes. Specificity of the PCR amplification was checked with a melting curve analysis (from 70 to 94°C) following the final cycle of the PCR. PCR conditions were optimized for high amplification efficiency (≥95% for all primer pairs used). In addition, plasmids were diluted several times to generate templates from 10^6 to 10^3 copies and used for standard curves for estimation of copy number in each cDNA sample.

The analysis of data was performed using Rotor Gene software (version 6.1.81). The relative quantification of

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Table 1 Gene-specific primer pairs used for real-time RT-PCR

DD-PCR clone (bp)	Primer pair	Sequence (5′-3′)	RT-PCR product size (bp)
cDNA1 (706)	Forward	AAGGATCTCTGCTTGGATGG	205
	Reverse	CAAGCAGAACTCAAGGCTCA	
cDNA2 (451)	Forward	CTAGTCCCGACTACCCTTCG	210
	Reverse	TCGAGGCACATAATGCAGAT	
cDNA3 (1064)	Forward	TTTTCGCAGTGTGAGAACTAGG	515
	Reverse	TTCATTCAAAGCCGAAGTGT	
cDNA4 (629)	Forward	AGGACCGGGGAGCTCTATAA	502
	Reverse	TTGAATCACTTCCCCCAATC	
cDNA5 (447)	Forward	TTGTGGTGGAAGAAACTCCA	240
	Reverse	AATTGGCTCCTGGTTGAATC	

bp = base pairs

transcript abundance of target genes in individual plant samples was determined by the $2^{-\Delta\Delta CT}$ method. Major changes of various genes relative to control were calculated for each replicate of each sample (Livak and Schmittgen 2001).

Amplifying and Cloning the Full-Length cDNA

Adaptor ligation-mediated PCR genomic walking To isolate the unknown 3′ regions of genes from genomic DNA, the modified protocol of adaptor ligation-mediated PCR genomic walking was employed (Mibus and Tatlioglu 2004). This technique enables the amplification a sequence situated between a primer annealing in a known region and an adjacent restriction site in an unknown region (Devon and others 1995).

About 2.5 μg of DNA was digested in a 15-μl reaction volume containing 5 U *Xba*I restriction enzyme, and 1 μl of corresponding buffer (MBI Fermentas Co.) overnight at 37°C. The reaction was deactivated by heating at 65°C for 10 min. *Xba*I splinker (5′-CTAGTCTTGGCTCGTTTTTT TTTGCAAAAA-3′) was annealed to splinktop (5′-CGAA TCGTAACCGTTCGTACGAGAATTCGTACGAGAATC GCTGTCCTCTCCAACGAGCCAAGA-3′) in a 10-μl reaction volume containing 20 mM Tris (pH 7.4) and 5 mM $MgCl_2$ at 90°C for 2 min before cooling at room temperature for 20 min. Then 3 μl of digested DNA and 6 μl of splinkerette *Xba*I were ligated after adding 10 U T4 ligase enzyme and buffer to a final volume of 20 μl and left overnight at 15°C according to the manufacturer's instructions. A nested PCR was performed for amplification of the desired fragment. The primer splk0 (5′-CGAATCGTAACCGTTCG TACGAGAA-3′) was applied in the first PCR together with a gene-specific primer (5′-AAGGAGACACCGTCATCG TC-3′). The second PCR was done by splk1 primer (5′-CG AATCGTAACCGTTCGT ACGAGAA-3′) together with the nested gene-specific primer (5′-TTCAACCAGGAGC-CAATTTC-3′) and using the first PCR product as template.

Rapid amplification of cDNA ends (RACE) To isolate full-length cDNA of *RhLAC*, the 5′/3′ RACE Kit (Roche) was used according to the supplier's instructions. The first cDNA strand was synthesized using oligo-dT anchor primer in a reverse-transcription reaction described above. Five percent of the cDNA was then used as a PCR template. To amplify the 3′ end of cDNA, nested PCR used an anchor primer together with a gene-specific primer 1 (5′CAGGAGAGTG GTGGAAGGAA3′) in the first PCR and gene-specific primer 2 (5′ATTCCTCCGCTTTCCTCATT3′) in the second PCR. Both gene-specific primers were derived from DNA isolated from ligation-mediated PCR genomic walking.

DNA extraction and Southern blot hybridization Genomic DNA was isolated from *Rosa hybrida* L. cv. Lavender and diploid rose genotype 94/1 using the modified hexadecyltrimethylammonium bromide (CTAB) method (Xu and others 2004). For genomic Southern blots, 10 μg of genomic DNA from each sample were digested with *Xba*I, *Eco*RV, *Eco*RI restriction enzymes overnight and run together with the DNA molecular weight marker III (Dig-, 0.56–21.2 kb; Roche, 1 μg) on 0.8% agarose gel. DNA was blotted onto a positively charged nylon membrane (Hybond N^+, Amersham Pharmacia Biotech Co.) with a vacuum blotter (Bio-Rad). The probe was a PCR-amplified 943-bp *RhLAC* fragment, using the plasmid containing the gene as a template and labeled with DIG dNTP (Roche). Membrane hybridization, posthybridization washing, and detection were performed as described in Sriskandarajah and others (2007).

Results

Cloning of Differentially Expressed mRNAs

In this study, 312 combinations of primers were tested, allowing detection of approximately 1910 fragments ranging from 100 to 1200 bp. With a total of 12 arbitrary

Table 2 List of ethylene-upregulated cDNA sequences, their size, *e* value, and homology to the sequences available at GenBank

cDNAs	size (bp)	Tissue	Homology	*e* value
cDNA-1	706	LP	*Cucumis melo* UDP-galactose/glucose pyrophosphorylase (ABD59006)	8e−40
cDNA-2	437	LP	*Arabidopsis thaliana* HB-1 (homeobox-1); transcription factor (NP_174164)	2e−25
cDNA-3	1064	BP	*Sypripedium parviflorum var.pubescens* trehalose-6-phosphate synthase/phosphatase (AAN86570)	2e−31
cDNA-4	629	BP	*Solanum demissum* kelch repeat-containing F-box family protein (AAT40540)	2e−90
cDNA-5	447	LP	*Zea mays* putative laccase (CAJ30499)	1e−08

LP = leaf petiole; BP = bud pedicel

primers in combination with 26 anchoring primers, 88 gene fragments were found to be upregulated, whereas 72 were downregulated in response to ethylene treatment compared to the control. To evaluate the authenticity of upregulated fragments, the PCRs were repeated using corresponding primers. Finally, 17 cDNA fragments that were differentially expressed following ethylene treatment were cloned and sequenced. Seven cDNA bands did not show any significant similarity to sequences existing in the database and five bands showed similarity to bacterial, fungal, and human genomes. Among the five expressed cDNAs, two were present in bud pedicels and three appeared in leaf petioles from ethylene-treated samples (Table 2).

Ethylene Induction of New cDNA Clones

The expression pattern of each cDNA was analyzed by real-time PCR using a gene-specific primer pair based on the nucleotide sequence of each clone (Table 1). Expression levels were rated relative to values expressed in control samples that were rated as 1. All specific genes were expressed more than twofold in pedicels compared to control (Figure 1). Following ethylene treatment, expression of cDNA-5 increased 54.2 times and cDNA-4 15.3 times compared to control. In petiole samples, except for cDNA-5 which increased 9.7-fold, cDNA-1 to cDNA-4 were expressed less than twofold compared to the untreated sample. The relative expression level of cDNA-5 in pedicels was five times higher than in petioles (Figure 1). Fragments of cDNA-5, in which expression levels were strongly affected by ethylene treatment, were selected for further analysis. Sequence analysis and database searching for the cDNA clone-5 indicated an approximate 77% homology with putative laccase in *Zea mays* (accession No. CAJ30499) and 51% homology with laccase 14 (accession No. NP_196498) from *Arabidopsis thaliana*. This 447-bp partial cDNA isolated from cDNA-5 is henceforth termed the *Rosa hybrida* Laccase (*RhLAC*) gene.

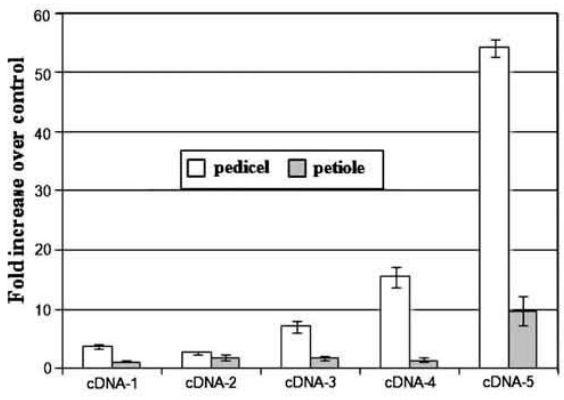

Fig. 1 Relative expression of the isolated cDNA genes induced in pedicel and petiole of rose after 72 h of ethylene treatment. The fold-change expressions of the isolated genes were calculated relative to the untreated sample as control after normalization to the β-actin gene. Expression level in untreated samples is defined as 1. Vertical bars represent $\pm$ standard deviations ($n = 3$)

Cloning and Sequence Analysis of the the Full-Length *RhLAC* cDNA

By comparing the cDNA sequence of *RhLAC* with its homologous sequences, it was shown that this cDNA fragment is the 5′ end of mRNA. To clone the whole mRNA sequence, 3′-RACE amplification was carried out. At first, PCR genomic walking was gained and a 460-base fragment was isolated that shared 28 bp with the previously isolated cDNA. The intron inside the fragment contained 97 bp. By constructing specific primers from this isolated fragment, 3′-RACE amplification PCR was done and finally a 1139-bp fragment was sequenced. The full-length sequence of this gene (accession No. EU603403) was 2005 bp which encoded a 63-kDa polypeptide (573 amino acids). Three putative conserved domains of the multicopper oxidase family were detected in the amino acid sequence levels of this fragment. When the deduced amino acid sequence of the RhLAC protein was compared to the protein databases, it showed similarity to a number of polypeptides. Alignment of the deduced amino acid sequence of this gene (Figure 2) indicated a 58% identity to putative laccase in *Zea mays* (accession No. CAJ30499) and 56 and 53% identity to laccase 15 (accession No. NP_199621) and laccase 14 (accession No. NP_196498) in *Arabidopsis thaliana*, respectively.

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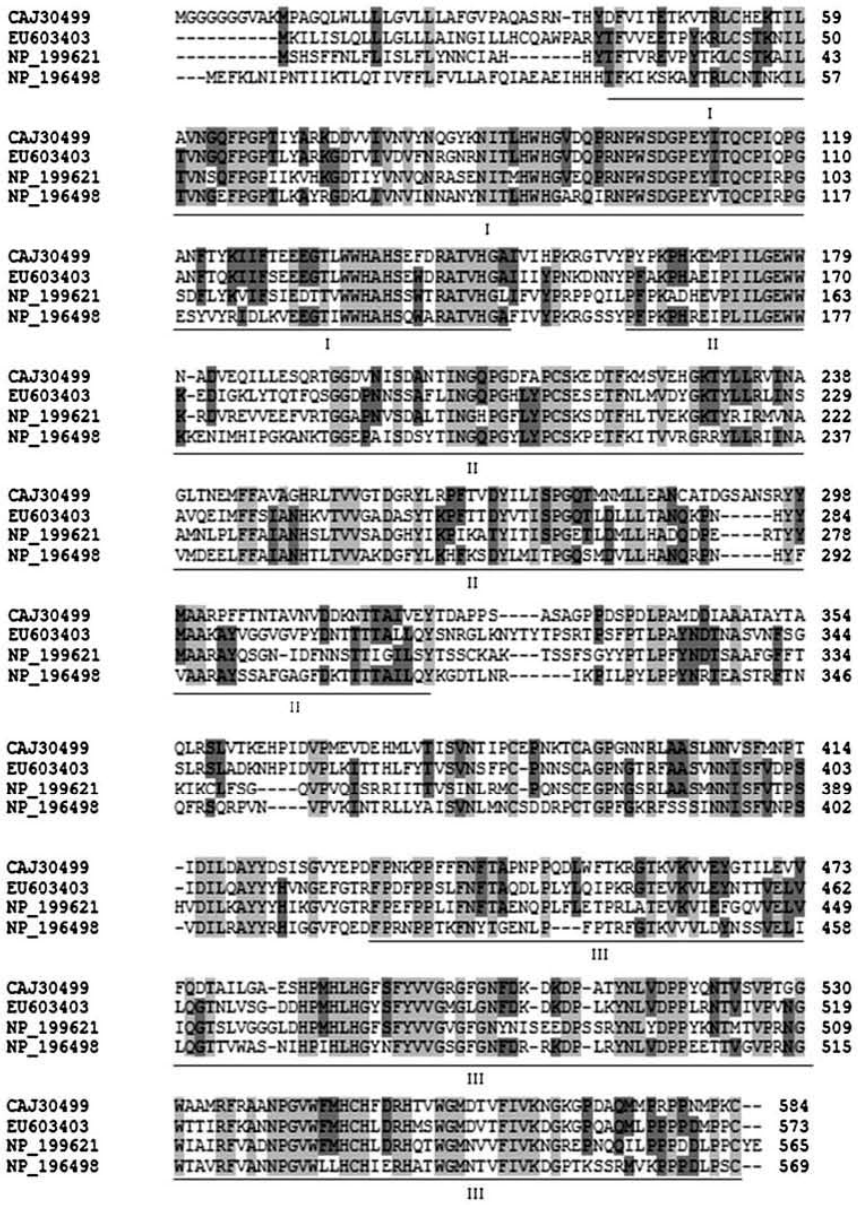

Fig. 2 Comparison of the deduced amino acid sequence laccase from *Rosa hybrida* (accession No. EU603403) with putative laccase from *Zea mays* (accession No. CAJ30499), copper ion binding from *Arabidopsis thaliana* (accession No. NP_196498), and TT10 (TRANSPARENT TESTA 10) laccase from *Arabidopsis thaliana* (accession No. NP_199621). Amino acid sequences were aligned and compared by the CLUSTAL W (1.83) program. Residues that are identical in all the sequences are shaded light gray, and those identically shared in *Rosa hybrida* and two of three sequences are shown in black boxes. Alignments of the three putative conserved domains of the multicopper oxidase family have been underlined and shown as I, II, and III. The sequence of probes is extended from amino acid number 354 573

The relationship between RhLAC protein and 17 sequenced laccases in *Arabidopsis* was determined (Figure 3) using a phylogenetic tree based on the CLUSTAL W program (EMBL) (Higgins 1994). According to the phylogenetic tree, RhLAC is closer to AtLAC15 and AtLAC14 than other AtLACs proteins.

Evaluation of *RhLAC* Homologs in the Rose Genome

Southern blot hybridization analysis of *Rosa* genomic DNA indicated a restriction pattern that was consistent with the RhLAC protein being encoded by multiple gene copies (Figure 4). Specifically, analysis of the *RhLAC* cDNA sequence including one intron (87 bp) region in the probe sequence revealed the presence of two internal *Xba*I and *Eco*RV sites. Digestion of genomic DNA with the enzyme *Eco*RI resulted in a complex pattern of at least 12 and 10 hybridizing bands in the Lavender (T) and diploid rose genotype (D), respectively. Similar patterns occurred after digestion with the enzymes *Xba*I and *Eco*RV when genomic DNA was probed with the 943-bp internal fragment from the *RhLAC* gene which contains one of three putative

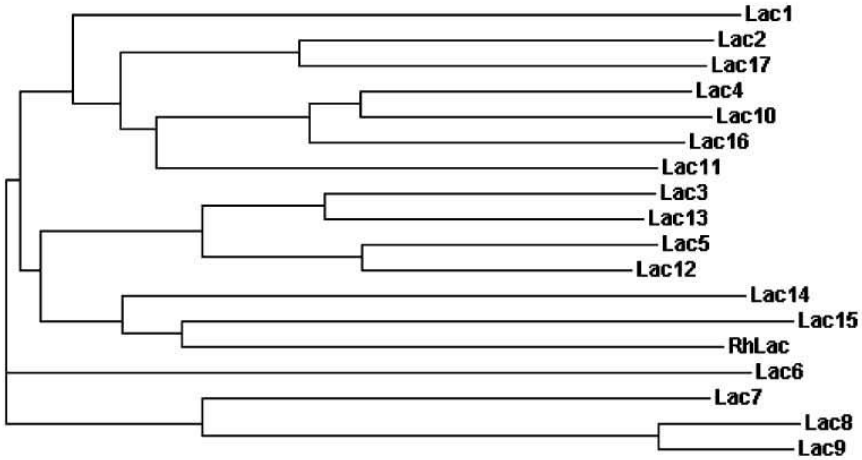

Fig. 3 Phylogenetic analysis of *RhLAC* with 17 laccases isolated from *Arabidopsis thaliana*. Sequence alignment analysis was performed using a CLUSTAL W method. The following are the corresponding accession numbers for each protein: AtLAC1 (NP_173252), AtLAC2 (NP_180477), AtLAC3 (NP_180580), AtLAC4 (NP_565881), AtLAC5 (NP_181568), AtLAC6 (NP_182180), AtLAC7 (NP_187533), AtLAC8 (NP_195724), AtLAC9 (NP_195725), AtLAC10 (NP_195739), AtLAC11 (NP_195946), AtLAC12 (NP_196158), AtLAC13 (NP_196330), AtLAC14 (NP_196498), AtLAC15 (NP_199621), AtLAC16 (NP_200699), AtLAC17 (NP_200810). The phylogenetic tree was generated based on ClustalW alignment

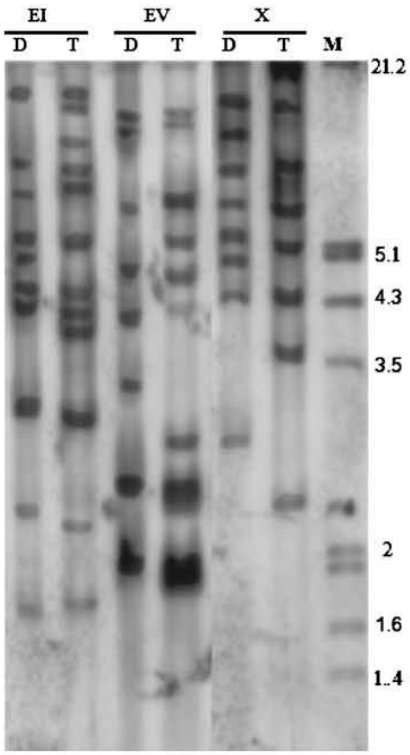

Fig. 4 Southern blot hybridization analysis of the *RhLAC* gene. The genomic DNA of diploid rose genotype 94/1 (D) and tetraploid *Rosa hybrid* L. cv. Lavender (T) were digested with *Eco*RI (EI), *Eco*RV (EV), and *Xba*I (X). M = DIG-labeled DNA Molecular Weight Marker III

conserved domains of the multicopper oxidase family (Figure 2). The *RhLAC* probe hybridized with many *Xba*I and *Eco*RV fragments of the rose genomic DNA, and at least eight bands associated with the fragments were detected in each digest (Figure 4). The combined results suggested that *RhLAC* was a multicopy gene family.

Expression of the *RhLAC* Gene in Different Tissues and Over Time

Because the expression level of the *RhLAC* gene was markedly higher than other clones, expression of this gene was studied in pedicel and petiole tissue 24, 48, and 72 h after ethylene treatment and in specific organs after 72 h. The highest relative expression of *RhLAC* occurred in pedicels was about 101-fold that of controls after 24 h, whereas in petioles it reached approximately 32.6-fold after 48 h (Figure 5). Twenty-four hours after ethylene treatment a decreasing gradient occurred in the relative expression of *RhLAC* in pedicels. In petioles, the mRNA level for *RhLAC* was detectable 24 h after treatment but showed a high level of induction after 48 h. Relative expression increased slowly from 26.9-fold to 32.6-fold after 48 h and then decreased to 9.7-fold after 72 h (Figure 5).

The highest relative expression of *RhLAC* occurred in the leaf abscission zone that included small parts of the petiolar pulvinus (LANZ), the bud abscission zone with a small segment of pedicel (BANZ) and petals, respectively (Figure 3). The lowest expression existed in leaf blades, petioles, and pedicels. The relative expression was

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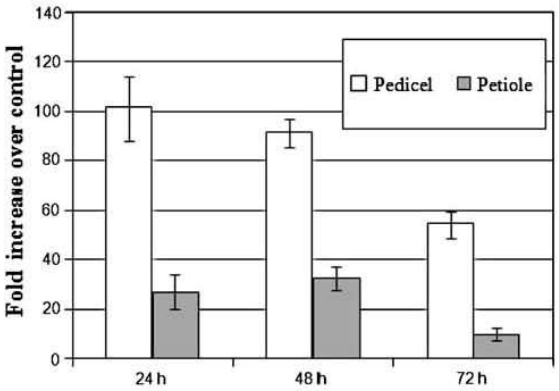

Fig. 5 Relative expression of the *RhLAC* gene induced in pedicel and petiole of rose after 24, 48, and 72 h of ethylene treatment. The fold-change expression of the isolated gene was calculated relative to the untreated sample as control after normalization to the β-actin gene. Expression level in untreated samples is defined as 1. Vertical bars represent $\pm$ standard deviations ($n = 3$)

different in various parts of leaf; an increased gradient of expression occurred from leaf blade to petiole and to the LANZ leaf abscission zone.

Discussion

The differential display RT-PCR technique (Liang and Pardee 1992; Liang 2002) is used extensively to identify and analyze expression patterns of uncharacterized genes in different plant species (Yamazaki and Saito 2002; Liu and Baird 2003). Thus, genes involved in physiologic events, including signal transduction pathways, stress responses, and secondary metabolism, have been isolated and characterized. Using this technique, Yamazaki and Saito (2002) listed some isolated genes that were expressed in response to various physiologic conditions such as dormancy, cell cycle, programmed cell death, and reaction to plant growth regulators.

This differential display (DD) method enabled identification of five partial cDNA transcripts in roses as being differentially expressed in response to exogenous ethylene treatment (Table 1). In the DD RT-PCR screen, 71 of 88 (80%) of DD amplicons were false positives, a serious drawback of this technique (Debouck 1995).

The homologs of cDNA-1 and cDNA-2 are *Cucumis melo* UDP-galactose/glucose pyrophosphorylase (accession No. ABD59006) and *Arabidopsis thaliana* HB-1 (homeobox-1) transcription factor (accession No. NP_174164), respectively. The exact role of the enhanced expression of these cDNAs under ethylene treatment is not understood. The cDNA-3 is a homolog to a gene that encodes the trehalose-6-phosphate/phosphatase protein in *Sypripedium parviflorum* and *Arabidopsis*. Trehalose is well known for its notable stress protection properties and it accumulates in vegetative cells under stress conditions (Van Dijck and others 2002). The homologs of cDNA-4 are found in *Solanum demissum* (accession No. AAT40540) and *Arabidopsis thaliana* (accession No. NP_178390) for kelch repeat-containing F-box family protein. F-box proteins are subunits of E3 ubiquitin ligase complexes that specify the protein substrates for degradation via the ubiquitin pathway (Sun and others 2007). It is suggested that expression of these genes following ethylene treatment might be related to stress conditions induced by ethylene or to destruction of proteins in programmed cell death.

The analysis of the *RhLAC* coding sequence (cDNA-5) showed 77% identity to putative laccase in *Zea mays* and the degree of homology after cloning the total length of the cDNA was 58%. The total cDNA length was shown to be 56 and 53% identical to LAC15 (accession No. NP_199621) and LAC14 (accession NP_196498) in *Arabidopsis thaliana*, respectively. We assumed that the unknown cDNA-5 is a putative laccase in *Rosa hybrida* and so designated the gene *RhLAC*.

In relation to the phylogenic tree, the RhLAC protein is close to AtLAC15 and AtLAC14. Different physiologic functions are ascribed to AtLAC15 (transparent testa) in *Arabidopsis* plants, including involvement in oxidative polymerization of flavonoids in the seed coat (Pourcel and others 2005; Cai and others 2006), and lignin synthesis and root elongation (Liang and others 2006a). No information exists allowing clarification of AtLAC14 function.

Southern blot hybridization analysis indicated that multiple copies of the *RhLAC* gene exist in *Rosa* species; similar, to laccases in *Zea maize* that belong to a multigene family (Caparros-Ruiz and others 2006). In higher plants, genome sequence studies have shown that multicopper oxidases related to laccase are widely distributed as multigene families (McCaig and others 2005).

Relative expression of the *RhLAC* gene in leaf blade and petiole was lower than in the LNAZ sample (Figure 6) because *RhLAC* expression occurred also in the corresponding control samples of these organs. Such organ- or tissue-specific gene expression analyses are important and need further intensive investigation. It is possible that it is related to endogenous ethylene produced in different tissues that induced expression of *RhLAC* in nontreated samples. A comparison between control plants grown in glass chambers and those grown in a greenhouse indicated that *RhLAC* was expressed at a lower rate in the former with virtually nothing produced in the latter, perhaps a result of high-humidity stress in chamber-grown plants (data not shown).

More than 1500 ethylene-responsive genes were identified by microarray analyses in mature mandarin and *Arabidopsis thaliana*, but less than half of these genes were

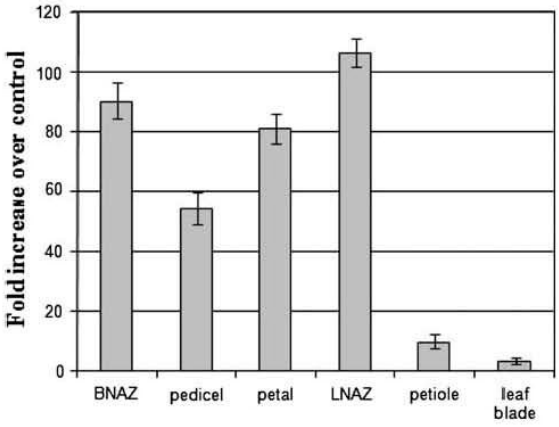

Fig. 6 Relative expression of the *RhLAC* gene expressed under 72 h of ethylene treatment in the bud abscission zone together with 2 mm of the proximal region of floral bud pedicel (BANZ), pedicel, petal, leaf abscission zone together with 2 mm of petiolar pulvinus (LANZ), petiole, and leaf blade. The fold-change expression of the isolated gene was calculated relative to the untreated sample as control after normalization to the β-actin gene. Expression level in untreated samples is defined as 1. Vertical bars represent $\pm$ standard deviations ($n = 3$)

upregulated by ethylene (Zhong and Burns 2003; Fujii and others 2007). However, the present work is the first report on upregulation of laccase following ethylene treatment.

Laccase is a multicopper-containing glycoprotein enzyme containing four copper atoms per protein molecule (Makino and Ogura 1971; Solomon and others 1996). Laccase activity has been detected in many plants and microorganisms and is involved in lignin biosynthesis and degradation, wound-healing as part of a herbivore or pathogen defense response, pigment formation, detoxification, and production of antimicrobial compounds in fungi active against various bacterial species (Solomon and others 1996; Mayer and Staples 2002; McCaig and others 2005). In spite of much research, genetic evidence for the role of laccase in plants is not clear. Recent studies on a number of laccase mutants showed that laccases have several different functions. For example, a mutant for *AtLAC2*, *lac2*, promoted root elongation under dehydration conditions, and mutants for *AtLAC8* and *AtLAC15* showed early flowering and changed seed color, respectively (Cai and others 2006). Although laccase plays a major role in the lignification process, laccase-like multicopper oxidases (LMCOs) exist in various plant organs of *Arabidopsis* that contain no significant amounts of lignin. LMCO expression in young roots suggests that these gene products participated in other physiologic functions as well as lignin deposition (McCaig and others 2005).

It is likely that laccase is expressed following stress in plants. The transcript level of laccase mRNA increased under abiotic stress (Liang and others 2006b), salinity stress, and after application of abscisic acid (Wei and others 2000). Relationships exist between abscisic acid (ABA) and ethylene, especially in abscission. ABA induced ethylene production and subsequently abscission in different organs in rose (Mayak and Halevy 1972; Müller and others 2001b) and other plants (Suttle and Hultstrand 1993; Chen and others 2002). Application of ABA to *Phaseolus vulgaris* promoted ethylene production and induced explant abscission (Jackson and Osborne 1972). At the transcription level auxin-induced ethylene-upregulated ABA biosynthesis occurred in cleavers (*Galium aparine*) through stimulated cleavage of xanthophylls to xanthoxin, catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (Kraft and others 2007).

Induction of defense mechanisms, especially the wound response of plants, can occur as a result of several signaling pathways, including those involving jasmonic acid (JA), salicylic acid, ethylene, and ABA (Ecker and Davis 1987; O'Donnell and others 1996; Stotz and others 2000). The central role of ethylene in inducing wound-responsive genes has been demonstrated, because in the presence of ethylene inhibitors, other signaling pathways involving systemin or JA were unable to induce expression of the proteinase inhibitor (Francia and others 2007). Therefore, in addition to its involvement in any ethylene response and stimulation of lignin biosynthesis (Solomon and others 1996; Mayer and Staples 2002; McCaig and others 2005), the laccase gene might be involved in wound-healing processes occurring after organ abscission. Organ detachment creates open wounds on the abscission zone and laccase may be involved in repair of damaged parts by regeneration of protoplasts and lignin deposition in the wounded regions (Mayer and Staples 2002).

In conclusion, five partial cDNAs expressed under ethylene treatment were isolated by DD RT-PCR and the full length of the *RhLAC* gene was isolated using RACE amplification. Insufficient material was available to explore the role of laccase expression under ethylene treatment. Further research on laccase expression and characterization of laccase functions in the abscission process will enable better understanding of the roles that laccase might have in plant response to stress conditions in general and to ethylene in particular.

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Characterization of ethylene-induced organ abscission in F1 breeding lines of miniature roses (*Rosa hybrida* L.)

Noorollah Ahmadi, Heiko Mibus and Margrethe Serek*
Faculty of Natural Sciences, Institute for Ornamental and Woody Plant Science, Leibniz University of Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany
*Author for correspondence (e-mail: Serek@zier.uni-hannover.de; phone: +49-511-7622657; fax: +49-511-7622654)

Abstract
Miniature potted roses are sensitive to ethylene and respond to it by bud and leaf abscission, leaf yellowing and/or flower senescence. The goal of this study was to develop a selection strategy for characterization of ethylene sensitivity in rose breeding lines. Two hundred and thirty-three F1 genotypes were obtained from a reciprocal crossing of 'Lavender' and 'Vanilla' cultivars, and evaluated in response to ethylene treatment. Based on ethylene-induced organ abscission, less sensitive and high sensitive genotypes were selected for physiological and molecular studies compared with the parent plants. After 1 week of ethylene treatment, leaf chroma values and chlorophyll degradation were increased in high sensitive genotypes, much more than in low sensitive genotypes. However, after 2 weeks of ethylene treatment, low sensitive genotypes showed no different in chroma value, but chlorophyll content decreased in 2 out of 3 genotypes. Expression of ethylene receptor genes (*RhETR1/3*) and signal transduction genes (*RhCTR1/2*) was not correlated with ethylene sensitivity of the investigated genotypes. Our previous research showed that laccase expression increased after ethylene treatment in 'Lavender'. The relative expression of laccase (*RhLAC*) was evaluated with considering the relation of lignin deposition and abscission process. Ethylene-induced expression of *RhLAC* in high sensitive genotypes was increased more strongly than in low sensitive genotypes. The lowest level of *RhLAC* transcript was accumulated in pedicels of genotype that showed the lowest bud abscission. Expression of the laccase gene by ethylene may be related to the abscission process.

Keywords: *CTR*; *ETR*; F1 hybrid; laccase; leaf yellowing; postharvest quality; *RhLAC*

1. Introduction

Miniature roses are popular potted plants that are very attractive to consumers. Satisfactory display quality and postharvest longevity are key factors for acceptance of miniature roses in international trade, especially for long distance exports. From a commercial perspective, leaf yellowing and abscission of floral organs reduce marketability of many potted plants, including roses. These negative quality effects are caused mainly by endogenous or exogenous ethylene (Serek, 1993; Serek et al., 1994; Müller et al., 2001). Therefore, because of these harmful effects of this gaseous hormone, it would be beneficial to eliminate ethylene, or to prevent its action, during marketing of potted rose plants. To improve postharvest quality of miniature roses previous research has focused on inhibiting ethylene action with silver thiosulfate (STS) (Serek, 1993; Hassan et al., 2004) or 1-methylcyclopropene (1-MCP) (Serek et al., 1994; Sisler and Serek, 1999; Buanong et al., 2005). However, because of silver toxicity and limited period of 1-MCP activity in plant tissue (Sisler and Serek 2001, 2003; Sisler et al. 2003) other possibilities for increasing shelf life of potted plants should be considered to reduce sensitivity to ethylene and thus satisfy consumers' demands.

Müller et al. (1998) investigated 15 commercially grown cultivars from two different breeding lines to find the best candidates for a future breeding program. Response of potted roses to exogenous ethylene treatment, and also in an ethylene free environment, varied dramatically among cultivars. The postharvest attributes of cultivar 'Vanilla' from the Kordes breeding

line differed significantly from other cultivars evaluated. 'Vanilla' had an excellent shelf life and appeared to be almost ethylene insensitive to ethylene. Thus, the potential exists to develop a breeding program to utilize this genetic variation among cultivars to create new miniature roses with extended shelf life. The classical approach for postharvest improvement is to select suitable cultivars that retain a high display quality when maintained in environment containing ethylene. Huge populations of F1 breeding lines are evaluated each year for appearance attributes, such as flower color and shape. For instance, about 50,000 F1 breeding lines are produced annually at W. Kordes' Söhne Rosenschulen GmbH & Co KG, Sparrishoop, Germany (personal communication from T. Proll, Research Director). Therefore, it is necessary to develop efficient selection strategies for fast selection of lines with high postharvest quality. By investigating differences between the highest and the lowest ethylene sensitive genotypes, at molecular and physiological levels, it should be possible to reduce the time needed for the selection process.

Ethylene synthesized in a plant, or applied exogenously, affects expression of receptors and genes involved in the ethylene signal transduction pathway. Ethylene perception is the first step of involving ethylene in transcription activation. In rose plants, as in *Arabidopsis,* ethylene is perceived by a family of receptors similar to the bacterial two component system (Müller et al., 2000b). Müller et al. (2000a and 2000b) investigated the molecular basis for ethylene resistance in 'Vanilla' in comparison to 'Bronze', a cultivar with high ethylene production and sensitivity, and a short postharvest life. Müller et al (2000) suggested that differences in display quality may be related to differential expression of receptors. Expression level of the ethylene receptor *RhETR1* transcript in flowers of the short-lived 'Bronze' was higher than in 'Vanilla', and treatment with ethylene for 3 days increased the *RhETR1* transcript in flowers of both cultivars (Müller et al., 2000a). Expression of the *RhETR2* transcript increased markedly after ethylene treatment in 'Bronze', but not in 'Vanilla' (Müller et al., 2000b). The expression of *RhETR3* increased with time of ethylene exposure in both cultivars, but transcription accumulation of this gene in 'Vanilla' was much lower than in 'Bronze' (Müller et al., 2000b). Expression of *RhCTR1* and *RhCTR2*, acting downstream of the ethylene receptors in the signal transduction pathway, also increased in response to exogenous ethylene (Müller et al., 2002).

Recently, the ethylene-induced laccase gene (*RhLAC*) was isolated from *Rosa hybrida* 'Lavender' (Ahmadi et al., 2008). The *RhLAC* gene encodes for a putative protein of 573 amino acids containing three conserved domains characteristic of the multicopper oxidase family. The expression level of the *RhLAC* gene in 'Lavender' was significantly higher in ethylene treated samples compared with non ethylene treated samples (Ahmadi et al., 2008). The expression of the laccase gene is also increased by abscisic acid and salinity treatments (Wei et al., 2000; Liang et al., 2006). It is logical to assume that there are multiple copies of this gene in rose as well as in *Arabidopsis* and maize (Ahmadi et al., 2008; McCaig et al., 2005; Caparros-Ruiz et al., 2006).

The aim of this study was to develop selection strategies for screening plants differing in response to ethylene in order to reduce time and cost of breeding programs. We generated two F1 generations to create extensive genetic diversity encompassing the range of variability of ethylene sensitivity of the breeding progeny. These two generations consisted of two hundred and thirty-three offspring genotypes resulting from reciprocal crosses between 'Vanilla' and 'Lavender'. The 'Vanilla' cultivar is recognized as a genetic resource in a breeding program for improving the display quality through conferring ethylene insensitivity (Müller et al., 1998; T. Proll, Research Director at W. Kordes' Söhne Rosenschulen GmbH & Co KG, Sparrishoop, Germany, personal communication).'Lavender' has been reported as ethylene sensitive (Buanong et al., 2005). After selecting high and low ethylene sensitive plants, expression levels of ethylene receptors (*RhETRs*), genes involved in the

ethylene signal transduction pathway (*RhCTRs*), and laccase (*RhLAC*) were evaluated in pedicel and petiole samples.

2. Materials and methods

2.1. Plant materials

Rosa hybrida cv. 'Lavender' sensitive to ethylene, cv. 'Vanilla' with low sensitivity to ethylene, and two F1 generations were obtained from W. Kordes' Söhne Rosenschulen GmbH & Co KG, Sparrishoop, Germany. The two tetraploid rose F1 generations consisted of 166 genotypes (assigned as 42/) resulting from crossing 'Lavender' X 'Vanilla', and 66 genotypes (assigned as 76/) from crossing 'Vanilla' X 'Lavender'. Parent plants and their F1 generations were cloned as cuttings in an experimental greenhouse at the Leibniz University Hannover; under the following environment conditions: temperature 20°C/20°C (day/night) and 70% relative humidity (RH). Day length was extended to 16 h by SON-T lamps (Osram, 400W, Philips Co.) that supplied 350 µmol m^{-2} s^{-1}.

2.2. Exogenous ethylene treatment

To screen two F1 generation plants displaying high and low sensitivity to ethylene, three plants of each genotype were exposed to ethylene for 15 days. Plants were placed in a growth chamber and ethylene was injected to achieve a concentration of 1.5 (± 0.25) µl l^{-1}. Ethylene concentration was monitored twice 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}, injection temperature was 60 °C and column temperature was 60 °C. Abscission rate of leaves was evaluated on the 3rd day of ethylene treatment and continued every second day by counting the shedding leaves. The experiment was terminated after 15 days. For detail investigation, 4 genotypes from F1 generation 42/ and 3 genotypes from F1 generation 76/ were selected as high ethylene-sensitive genotypes based on leaf abscission rate on day 5 of ethylene treatment. On day 9 of ethylene treatment, 10 genotypes from F1 generation 42/ and 5 genotypes from F1 generation 76/ were picked as low sensitive genotypes based on leaf abscission. The selected F1 hybrid and parent plants were exposed to ethylene treatment conducted in a completely randomized design using three pots per treatment and three replications. Ethylene treatment and evaluation of leaf and bud abscission were carried out as described above. Three genotypes showing the lowest sensitivity and two genotypes with the highest sensitivity were chosen from F1 genotypes for further investigations.

2.3. Measurement of the color characteristics

Leaf samples were harvested from 3 genotypes with the lowest sensitivity for chroma value measurement after 0, 7 and 15 days of ethylene treatment. Leaf sampling of 'Lavender', 'Vanilla' and 2 sensitive genotypes was done after day 0 and 7 days. Representative leaf color measurements were carried out on the upper (adaxial) surface of fresh leaves using a Minolta Chroma Meter (Model CR-300, Minolta, Osaka, Japan) instrument. This tri-stimulus color analyzer, which consisted of a head with an 8 mm diameter measuring area, a diffuse illumination and a 0° viewing angle, was calibrated to a standard white tile before measurements were done and then was placed gently vertically on the leaflet. Three dimensional measurements of brightness from total black to pure white (L^*), red-green scale (a^*) and yellow-blue scale (b^*) were recorded (Robertson 1977). Chroma, which refers to the saturation of the colour, was calculated using the formulae: Chroma = $(a^2 + b^2)^{0.5}$

2.4. Chlorophyll determination

Leaf chlorophyll content was determined using 3 replicates of 4 leaf blades for each genotype. Chlorophyll content was analyzed according to Lichtenthaler (1987) by extracting in 80% ethanol for 10 min at 75°C, then repeated until all pigment was extracted from the samples. Absorption was measured

with a spectrophotometer at the wavelengths 700, 664 and 647 nm. Chlorophyll concentration was calculated using the following equation: $Chlorophyll_{a+b}$= 5.24 (A_{664}–A_{700}) + 22.24 (A_{647}–A_{700}). Where A_{700}, A_{664} and A_{647} were absorbances at the three wavelengths (Lichtenthaler, 1987).

2.5. RNA extraction and real-time PCR

Pedicel and petiole samples collected from 'Lavender', 'Vanilla', and F1 (1) genotypes (42/48, 42/131, 42/50) and F1 (2) genotypes (76/67, 76/74) were treated with 0 (control) or 1.5 µl l^{-1} (± 0.25) ethylene for 72 h. Total RNA was isolated using Invisorb^{R} Spin Plant RNA Mini Kit (Invitek Co.) according to the manufacturer's protocols. The first-strand cDNA synthesis was done as described by Ahmadi et al. (2008). To evaluate mRNA level expressions, real-time RT–PCR assays were performed using the Rotor Gene 3000 real time thermal cycler (Corbett Life Science Co.). In the PCR reaction mixture, gene-specific forward and reverse primer pairs (Table 1) were used as previously described (Ahmadi et al., 2008).

2.6. Statistical analysis

The data obtained in the ethylene test were subjected to analysis of variance (ANOVA) using software R multiple comparisons and Tukey test (R Development Core Team, 2008). The data analysis of real-time PCR was undertaken with Rotor Gene software (version 6.1.81). The relative quantification of transcript abundance of target genes in individual plant samples was determined by the $2^{-\Delta\Delta C}$T method. Major changes of various genes relative to control were calculated for each replicate of each sample (Livak and Schmittgen, 2001).

3. Results

3.1. Leaf and bud abscission

In the exogenous ethylene treatment experiment, two hundred and thirty-three genotypes showed marked differences in their postharvest quality as evaluated by leaf abscission. After 5 days, both F1 generations showed wide leaf abscission rates from less than 20% to more than 80% (Fig. 1). Average of leaf abscission in the F1 generation 42/ was 38.2% on day 5 of ethylene treatment and in the F1 generation 76/ it was 34.1%. Based on these data, 7 genotypes that lost nearly all leaves were selected as high sensitive genotypes. After 9 days of ethylene treatment, about twenty-five genotypes lost less than 80% of their leaves in both F1 generations. Of these twenty-five genotypes, fifteen genotypes with the lowest rate of leaf abscission were selected as having low sensitive to ethylene. 'Lavender', 'Vanilla', fifteen low sensitive and 7 high sensitive genotypes were used for further evaluation of leaf and bud abscission (data not shown). Finally, from the original two hundred and thirty-three genotypes of both F_1 generations, 3 genotypes with the lowest and 2 genotypes with high sensitivity to exogenous ethylene treatment were selected.

The three low sensitive genotypes (76/67, 42/48 and 42/131) had lost between 80-94 % of their leaves after 15 days of exposure to ethylene while the high sensitive genotypes (76/74 and 42/50) had lost all leaves on day 7 (Fig. 2). 'Lavender' and 'Vanilla' had 100% leaf abscission on days 9 and 11, respectively. By day 5, the high sensitive genotypes had dropped more than 90% of their leaves, whereas the lowest sensitive genotypes had lost between 5 and 40% of leaves.

Bud abscission rate of F_1 genotypes and their parents has been showed in Fig. 3. The high ethylene sensitive plants, genotypes 76/74 and 42/50, had lost 60 and 90% of their buds, respectively, after 5 days of ethylene exposure. The lowest sensitive genotypes lost less than 6% during the same period. Cultivars 'Lavender' and 'Vanilla' lost 88% and 70% of buds, respectively, during 7 days of ethylene treatment. One of the most ethylene insensitive genotypes, 42/48, lost 38% buds during the 15 day observation period, while the more sensitive genotypes and their parents had almost 100% bud abscission on day 9. In the 42/48 genotype, most bud pedicels were wilted and dried,

without development of an abscission zone and did not detach from branches.

3.2. Effects of ethylene on leaf color

Exposure to exogenous ethylene hastened the onset of visible leaf yellowing in the miniature rose plants investigated. Leaf chroma values increased in all genotypes except 2 low sensitive genotypes on day 7 of exposure to 1.5 µl l^{-1} ethylene. On day 15 of ethylene treatment, the chroma values did not change significantly in low sensitive genotypes, while genotypes 42/50, 76/74, 'Lavender' and 'Vanilla' were completely defoliated (Fig. 4). In low sensitive genotypes, chroma values increased slowly in genotype 76/67 during 7 days of ethylene exposure. There was no difference in chroma values in genotypes 42/48 and 42/131 during 15 days of treatment. In general, continuous exposure to exogenous ethylene stimulated significantly the increase of chroma value in parents and high sensitive genotypes than in low sensitive genotypes.

3.3. Chlorophyll content

Chlorophyll content in selected F_1 genotypes and parents was reduced after exposure to ethylene (Fig. 5). The rate of chlorophyll reduction was significantly difference in genotypes 42/50 and 76/74 versus genotypes 42/48, 42/131 and 76/67 after 7 day. Chlorophyll content of genotype 42/50 had decreased to 50% of the initial value. In low sensitive genotypes, chlorophyll content decreased in genotypes 42/48 and 42/131 through 15 days of treatment. Genotypes 42/50, 76/74, 'Lavender' and 'Vanilla' had lost all their leaves by day 15 of ethylene treatment. Chlorophyll degradation in genotypes 42/131 and 42/48 progressed significantly during the measurement period, while genotype 76/67 did not showed significant changes in chlorophyll content from day 7 to day 15 (Fig. 5).

3.4. Expression of RhETRs, RhCTRs family genes after 72 h ethylene treatment

Expression of ethylene receptor genes (*RhETR1*, *RhETR3*), genes involved in the ethylene signal transduction pathway (*RhCTR1*, *RhCTR2*) and the recently isolated laccase (*RhLAC*) gene were evaluated in parents, and in high and low ethylene-sensitive genotypes.

Expression of *RhETR1* in pedicel of the low sensitive genotype 76/67 showed a statistically significant difference (P= 0.05) versus genotypes 42/50 and 42/131(Fig. 6). In petiole of genotype 76/67, the relative expression of *RhETR1* was significantly up-regulated by ethylene treatment at P= 0.01, comparing to genotypes 42/48, 42/50 and 42/131 (Fig. 7). No significant difference found in relative expression of *RhETR3* in pedicel and petiole of studied genotypes. The relative expression of *RhCTR1* in pedicel of genotypes did not show any difference, but in the petiole of genotype 76/67 it was higher than genotypes 76/74 and 42/50 (P= 0.05). No significant difference of *RhCTR2* expression was observed in pedicel and petiole of genotypes.

3.5. Expression of laccase RhLAC gene after 72 h ethylene treatment

The relative expression levels of *RhLAC* were significantly different (P= 0.001) in petioles and pedicels of the low and high ethylene-sensitive genotypes studied. In general ethylene treatment induced greatly transcript accumulation of the *RhLAC* gene in pedicels and petioles of high sensitive (Fig. 8). The maximum of relative expression of *RhLAC* in low sensitive genotypes was about 6 fold in petioles and pedicels following ethylene application compared to untreated plants. The highest and the lowest levels of laccase transcript expressed in pedicel occurred in genotypes 76/74 and 42/48, respectively. In ethylene-treated petiole samples, laccase expression was lowest in genotype 42/131, while genotypes 42/50 and 76/74 showed the highest amount of laccase amplicon accumulation.

In the overall view, table 2 shows the ranking of genotypes in ethylene sensitivity. According to this ranking the highest ethylene sensitivity was set as 10 score. Genotypes

42/50 and 76/74 were placed on top with the highest scores, and genotypes 42/131, 42/48 and 76/67 got lowest scores. 'Lavender' and 'Vanilla' remained at third and fourth places.

4. Discussion

Since existing commercial cultivars of miniature roses come from crosses with different genetic backgrounds that have been selected based on their attractiveness and productivity, it is desirable to use the total F1 population to study plant reactions to ethylene. To create a wide variation in miniature potted rose germplasm for selection of genotypes with strong and weak reactions to exogenous ethylene, two hundred and thirty-three F1 hybrids were reciprocally crossed from 'Vanilla' and 'Lavender' cultivars. The miniature potted rose 'Vanilla' is known as a cultivar with excellent postharvest quality (Müller et al., 1998), in contrast to 'Lavender' which is an ethylene sensitive cultivar (Buanong et al., 2005). Offspring genotypes of two F1 generations showed various rates of leaf abscission after 5 and even through 9 days of ethylene treatment (Fig. 1). Leaf abscission rates of both F1 generations on day 5 of ethylene treatment suggest that there is no different effect of maternal or paternal inheritance on the ethylene sensitivity. However, the data presented indicate that ethylene plays an important role in display quality of miniature rose plants, consistent with results of Müller et al. (1998) and Buanong et al. (2005). These results suggest strongly that 'Vanilla' has excellent potential as a genetic resource for utilization as mother or father in any breeding program for improving the display longevity of miniature roses (Figs. 2 and 3).

'Vanilla','Lavender', and three genotypes with low and two genotypes with high sensitivity to ethylene were selected for further evaluation of important quality attributes including visual characteristics. Generally, exogenous ethylene accelerated leaf yellowing in all genotypes as indicated by increasing chroma values and decreasing chlorophyll content during 7 and 15 days of ethylene treatment (Figs. 4 and 5). Leaf yellowing increased sharply in high sensitive genotypes, but appeared more slowly on low sensitive genotypes leaves. Therefore, it is concluded that chlorophyll degradation leads to leaf yellowing and this is accelerated by exposure to ethylene. This is in agreement with observations on miniature rose plants and cut rose that ethylene accelerated yellowing (Serek et al., 1996; Cuquel et al., 2007). Inducing chlorophyll degradation and accelerating yellowing in leaves by ethylene was reported by Able et al. (2002, 2003). According to Alejar et al. (1988) both exogenous ethylene and endogenously produced ethylene accelerated yellowing in attached leaves of tobacco cultivars. Ethylene enhanced chlorophyllase activity and extended the period of high peroxidase activity in broccoli florets (Gong and Mattheis, 2003). Chlorophyllase cleaves chlorophyll into phytol and chlorophyllide (chlide), the Mg-porphyrin moiety of chlorophyll (Matile et al., 1996; Rodoni et al., 1997).

For in-depth insight into molecular differences in postharvest characteristics, the expression of ethylene receptors (*RhETRs*) and genes involved in the ethylene signal transduction pathway (*RhCTRs*) and laccase (*RhRhLAC*) were examined.

Accumulation of the *ETR1* transcript increased in pedicels and petioles of the long-lasting genotype, 76/67, comparing to genotypes 42/48, 42/50 and 42/131. Increased *ETR1* expression in genotype 76/67 agreed with findings in cut rose cv. 'Samantha' (Tan et al., 2006; Ma et al., 2006). Low expression of *RhETR1* in petiole of genotypes 42/48 and 42/50 is in contrast to results of Müller et al. (2000a and 2000b), who found that the expression of *RhETR1* was clearly increased under ethylene treatment in both the short flower life cultivar 'Bronze' and the long-lasting cultivar 'Vanilla'. The genes *RhCTR1* and *RhCTR2* were constitutively expressed in pedicels of all plants examined. In petioles, the expression pattern was different as *RhCTR1* increased in genotype 76/67, whereas *RhCTR2* expression did not change after ethylene treatment in all genotypes. These data are different from finding of increasing *RhCTR1* and *RhCTR2* in response to ethylene treatment in high and low

ethylene-sensitive cultivars (Müller et al., 2002). No clear effect of ethylene was found on the expression of fragments of *CTR* genes in cut rose cv. 'Kardinal' and 'Samantha' (Tan et al., 2006). These gene expression differences may be due to tissue-specific expression pattern of studied genes. In this study, the gene expression was evaluated in petiole and pedicel, while in other studies northern blot was applied on RNA isolated from flowers parts specially petal. Like Tan et al. (2006), we suggest further work to clarify the different results in miniature potted roses and in cut rose cv. Samantha and cv. Kardinal. Müller et al. (2000a and 2000b) suggested that differences in flower vase life of rose cultivars could be related to differences in receptor levels, but the present results do not support this theory. Some controversial results, similar to those reported here, have previously been reported on ethylene receptor expression. In peach, *ETR1* transcripts did not change in various tissues and developmental stages examined in response to applied propylene, an ethylene analogue (Rasori et al., 2002). In immature apple fruit, the expression pattern of *MdETR1*, *MdERS1*, and *MdCTR1* gene were related to tissue and/or population. Apple fruitlet populations were characterized by different abscission potentials (Dal Cin et al., 2005).

Considering these data, it seems that other factors play a role in regulation of plant response to ethylene. It is possible that the ETHYLENE-INSENSITIVE3 (EIN3)/EIN3-Like (EIL) family is necessary for plants to respond to ethylene, although the biochemical function of these nuclear proteins is still unclear (Solano et al., 1998). Loss-of-function mutations in EIN3 protein result in ethylene insensitivity in *Arabidopsis* (Ticman et al., 2001). Further studies are required to clarify the correlation between ethylene sensitivity and expression of genes acting as ethylene receptors and/or acting in MAPK cascades.

The abundance of the *RhLAC* gene in pedicels of highly sensitive genotypes was higher than in low sensitive genotypes. Expression of *RhLAC* was at lowest level in ethylene treated pedicels of the low sensitive genotype, 42/48, which showed the lowest abscission. Generally, expression of the *RhLAC* gene showed correlation with ethylene sensitivity as *RhLAC* expression was higher in highly ethylene sensitive genotypes than in low ethylene sensitive genotypes (Fig. 8).

In higher plants, various functions have been assigned to the laccase gene including: lignin biosynthesis (Solomon et al., 1996; Dean et al., 1998), wound-healing, pigment synthesis, and detoxification (Solomon et al., 1996; Mayer and Staples 2002; McCaig et al., 2005; Pourcel et al., 2005). To date, 17 laccase genes have been characterized from *Arabidopsis* with some laccase mutants linked to physiological functions including root elongation, earlier flowering and changing seed color (Cai et al., 2006). Numerous functions assigned to laccase might be related to different forms of this enzyme and/or also to its complex network of action.

Laccase expression under ethylene treatment (Ahmadi et al., 2008) or other biotic and abiotic stress conditions (Liang et al., 2006; Wei et al., 2000) may be related to plant defense mechanisms in reaction to stress conditions. Considering that organ abscission is induced by various abiotic and biotic stress conditions (Addicott, 1982), it is suggested that laccase may be involved somehow in the abscission process. In fact, it is probable that expression of laccase induced and/or promoted by ethylene may initiate a sign of abscission process in rose. This hypothesis is based on our previous research reporting isolation of laccase under ethylene treatment for the first time (Ahmadi *et al.*, 2008) and high expression of laccase in high ethylene-sensitive genotypes of present experiment. The findings of Agusti *et al.* (2008), who recently reported expression of laccase during the abscission of citrus leaves supports our hypothesis. Laccase participate in lignin synthesis and staining of abscission zone showed lignin deposition after ethylene treatment (Agusti *et al.*, 2008). Enhanced transcript accumulation of laccase in high sensitive genotypes compared to that in low sensitive genotypes suggests that this sign appeared greater in high sensitive than in low sensitive genotypes. Expression of laccase may be a molecular index for characterization of offspring plants in response to ethylene

during breeding program. This is, however, a very tentative suggestion that needs to be studied in detail.

In conclusion, ethylene accelerates leaf yellowing and abscission of floral buds and leaves in more sensitive genotypes than in low sensitive genotypes of miniature potted rose plants. Expression of *RhETR1*, *RhETR3*, *RhCTR1* and *RhCTR2* genes was not correlated with sensitivity to ethylene. In contrast, laccase expression was enhanced more in high sensitive genotypes than in low sensitive genotypes. Future studies will investigate and characterize phenotypes in different progenies to determine the main factors influencing ethylene sensitivity in miniature roses, *Rosa hybrida*.

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Table 1
Gene-specific primer pairs used for real-time RT-PCR

Gene	Accession number	Primer pair	Sequence (5´-3´)
*RhETR*1	AF394914	forward primer	TGGTATGAACCTTCAACTTTCTCA
		reverse primer	CGCATAGACTCTTCAAGAATAGCA
*RhETR*3	AF154119	forward primer	GGCTGGACTTATGGTCCTCA
		reverse primer	GGCTCACCAAAATCACCACT
*RhCTR*1	AY032953	forward primer	GGCTCTGATGTTGCTGTGAA
		reverse primer	TCAATGGCCTCAAAGATTCC
*RhCTR*2	AY029067	forward primer	TTCCTTCCAAGGGGAAGTCT
		reverse primer	CCCACTCCAAGCCAATTTTA
RhLAC	EU603403	forward primer	GAACCACCCCATTGATGTTC
		reverse primer	TGGCAGTCAGCATAAACCAA
Rhßactin	AB239794	forward primer	CCAGAAGAGCACCCTATACT
		reverse primer	ATGGCTGGAAGAGGACTT

Table 2

Ethylene-sensitivity ranking of genotypes

Genotypes	76/74	42/50	Lavender	Vanilla	42/48	42/131	76/67
Leaf abscission	10,0	9,9	9,6	7,6	6,2	3,2	7,4
Bud abscission	10,0	9,5	8,9	7,0	1,0	1,7	1,6
Chroma	7,4	8,6	10,0	4,8	1,6	1,3	2,5
Chlorophyll degradation	5,9	10,0	4,6	5,6	4,0	3,2	2,6
laccase pedicel	10,0	1,6	1,1	0,2	0,0	0,1	0,0
laccase petiole	4,1	10,0	2,0	1,1	0,7	0,3	0,6
Total	**47,5**	**49,7**	**36,2**	**26,2**	**13,6**	**9,7**	**14,8**

The highest ethylene sensitivity is 10 score. The data of leaf abscission, bud drop, chroma and chlorophyll degradation of day 7 are presented.

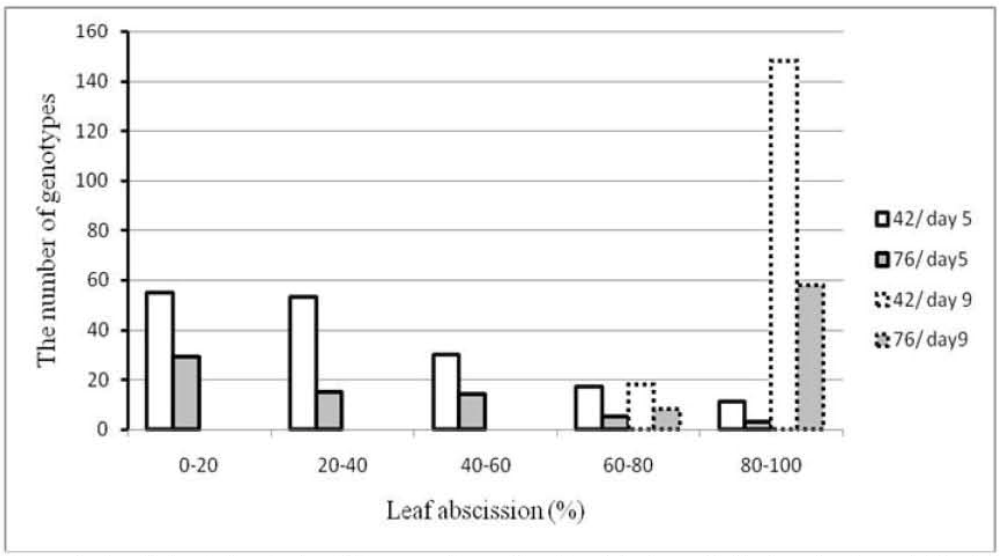

Figure 1. Leaf abscission in two F1 generations after 5 and 9 days of ethylene treatment. The F1 generation resulted from 'Lavender' X 'Vanilla' were assigned as 42/ and the F1 generation from 'Vanilla' X 'Lavender' were showed as 76/.

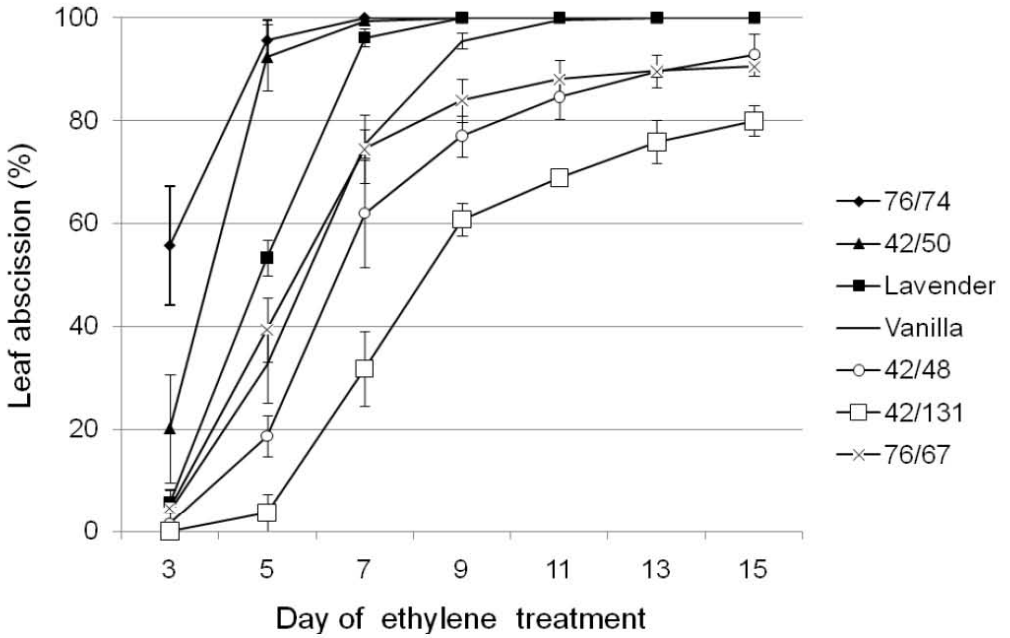

Figure 2. Leaf abscission in 'Vanilla', 'Lavender', and 5 F_1 genotypes of *Rosa hybrida* during 15 days exposure to 1.5 (± 0.25) $\mu l\,l^{-1}$ ethylene. Values are means of 3 replicates.

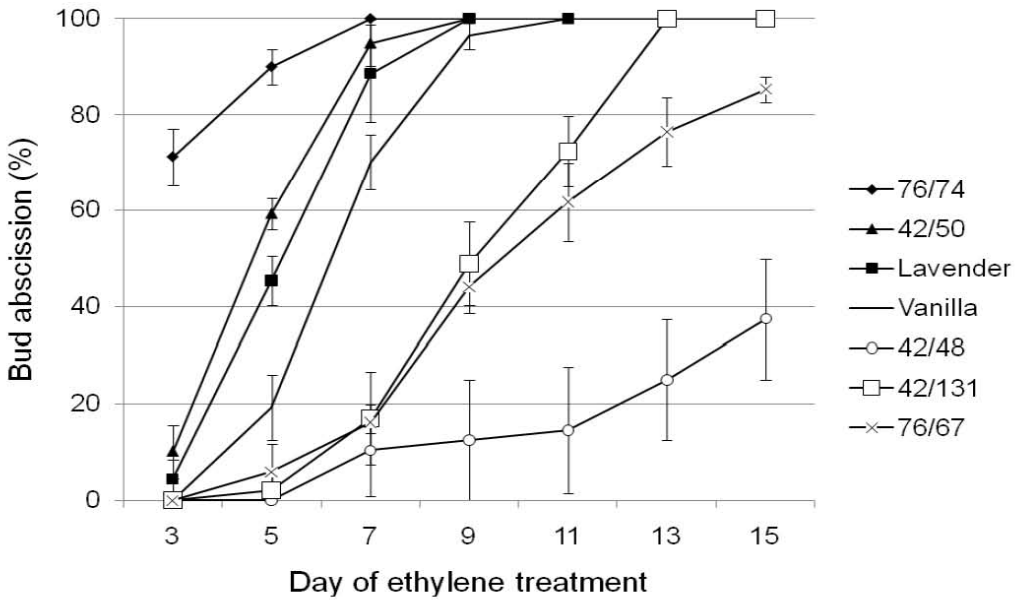

Figure 3. Bud abscission in 'Vanilla', 'Lavender', and 5 F_1 genotypes of *Rosa hybrida* during 15 days exposure to 1.5 (± 0.25) $\mu l\,l^{-1}$ ethylene. Values are means of 3 replicates.

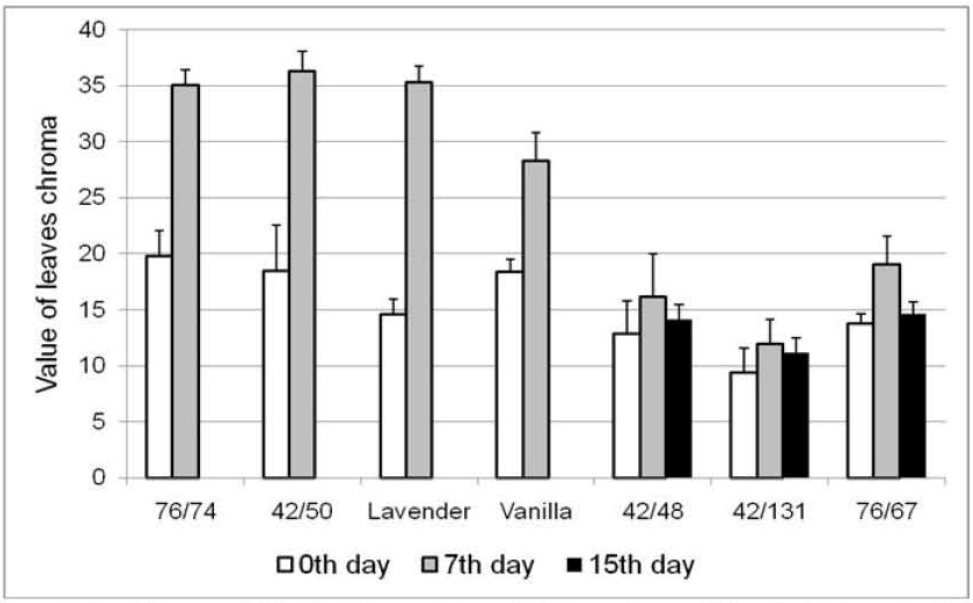

Figure 4. Leaf chroma of 'Vanilla', 'Lavender', and 5 F_1 genotypes of *Rosa hybrida* on day 0 (initial), 7 and 15 of exposure to 1.5 (± 0.25) µl l^{-1} ethylene.

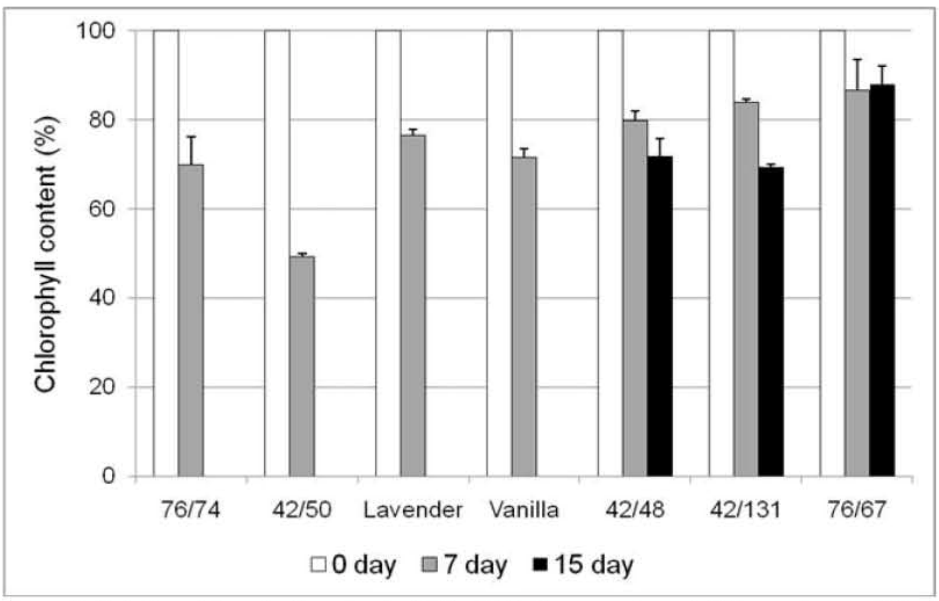

Figure 5. Relative chlorophyll content of 'Vanilla', 'Lavender', and 5 F_1 genotypes of *Rosa hybrida* on day 0, 7 and 15 of exposure to 1.5 (± 0.25) µl l^{-1} ethylene. The chlorophyll content of each genotype on day 0 was set to 100%.

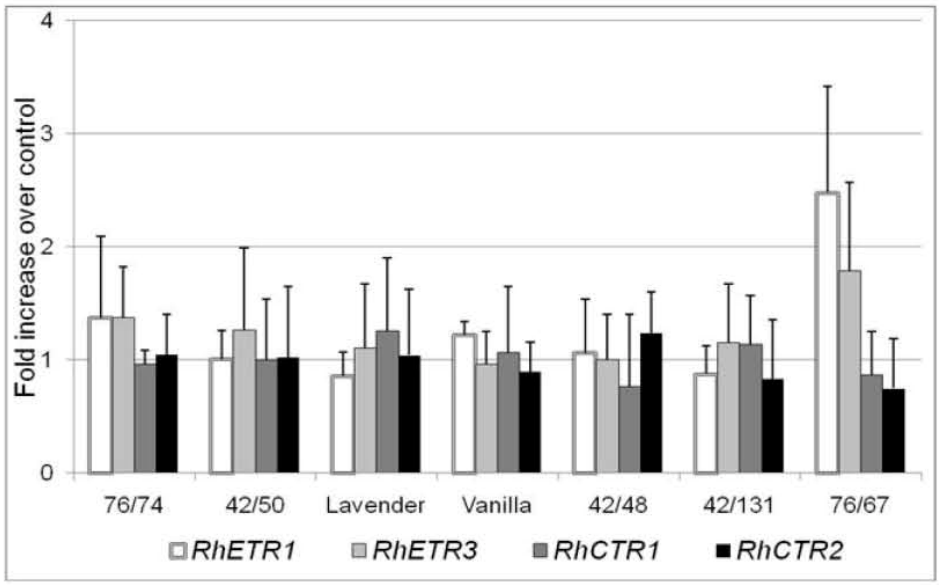

Figure 6. Relative expression of *ETR1*, *ETR3* ,*CTR1* and *CTR2* genes induced in the pedicel of *Rosa hybrida* after 72 h of ethylene treatment. The fold change expressions of genes were calculated relative to the untreated sample as control after normalization to the *Rhβ-actin* gene. Expression level in untreated samples is defined as 1. Vertical bars represent standard deviations (n=3).

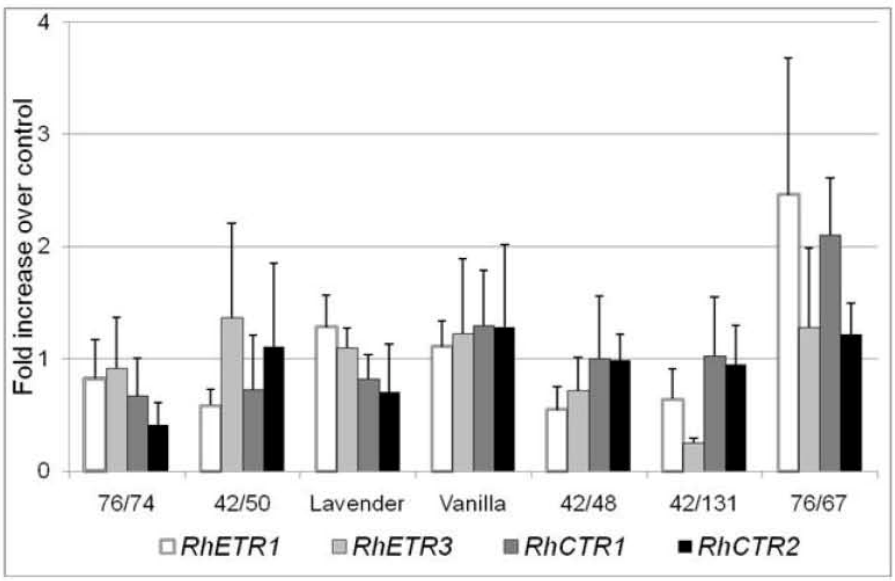

Figure 7. Relative expression of *ETR1*, *ETR3* ,*CTR1* and *CTR2* genes induced in the petiole of *Rosa hybrida* after 72 h of ethylene treatment. The fold change expressions of genes were calculated relative to the untreated sample as control after normalization to the *Rhβ-actin* gene. Expression level in untreated samples is defined as 1. Vertical bars represent standard deviations (n=3).

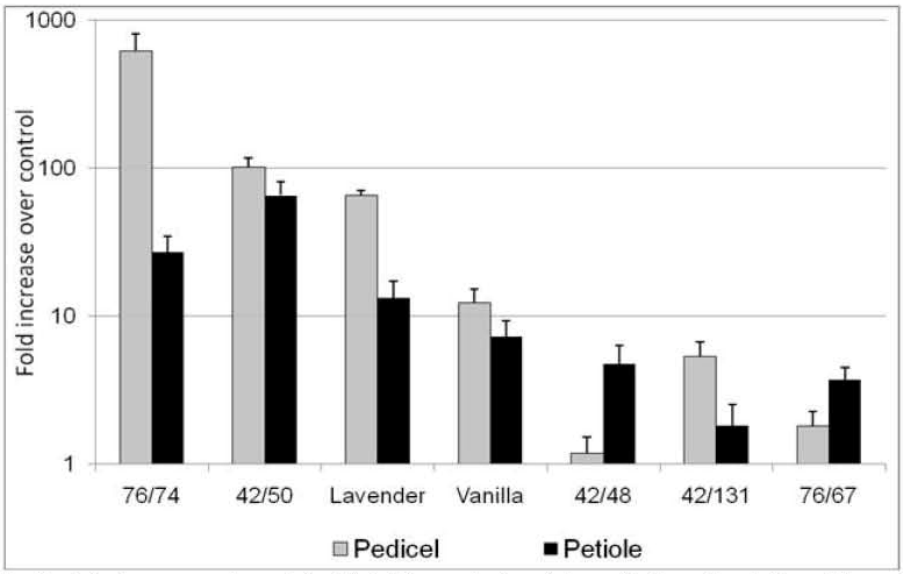

Fig. 8. Relative expression of the *RhLAC* gene induced in pedicels and petioles of *Rosa hybrida* after 72 h of ethylene treatment. The fold change expression of the *RhLAC* gene was calculated relative to the untreated sample as control after normalization to the *Rhβ-actin* gene. Expression level in untreated samples is defined as 1. Vertical bars represent standard deviations (n=3).

Functional analysis of rose laccase (*RhLAC*) gene in tobacco plant by virus-induced gene silencing technique

Noorollah Ahmadi, Heiko Mibus and Margrethe Serek*

Faculty of Natural Sciences, Institute for Ornamental and Woody Plant Science, Leibniz University of Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany
*Author for correspondence (e-mail: Serek@zier.uni-hannover.de; phone: +49-511-7622657; fax: +49-511-7622654)

Abstract

Virus-induced gene silencing (VIGS) is a powerful tool for gene functional analysis by interrupting gene function through RNA interference. The function of ethylene-induced laccase gene isolated from rose was evaluated in *Nicotiana benthamiana*. The plants size and total green leaves area per plant were decreased by laccase silencing and ethylene treatment. Ethylene treatment reduced significantly size and total leaves area in plants with silenced laccase compared to control plants. The numbers of leaves per plant were decreased by ethylene treatment. It is assumed that cell division and/or cell elongation were inhibited by laccase gene suppression.

Keywords: ethylene, gene silencing, laccase, miniature rose, virus vector

Introduction

The responses of plant to ethylene vary among the plants individual genotypes, especially among organ tissues. Studying of petal senescence of 93 plant species showed that the level of sensitivity to exogenous ethylene harmonized with the family relationship (Woltering and van Doorn, 1988). Flower abscission in most of 300 species showed high sensitivity to ethylene (van Doorn, 2002).

Ethylene increased the RNA levels of some genes involving in abscission zone including cellulase and pectinase enzymes (del Campillo and Bennett, 1996; Tucker *et al.*, 1988). In addition, chitinases and ß-1,3-glucanase, uronic acid oxidase and peroxidases, have been associated with abscission processes (Sexton *et al.*, 1985; Brown, 1997). In general, genes encoding proteins associated with cell-wall disassembly during fruit ripening, leaf abscission and leaf senescence, contain promoter elements regulated by ethylene (Bleecker and Patterson, 1997).

A large group of genes up-regulated by ethylene were characterized in *Arabidopsis thaliana* as well as in other species (Zhong and Burns, 2003; Fujii *et al.*, 2007). Recently, we isolated laccase gene from rose plant expressed under ethylene treatment (Ahmadi *et al.*, 2008).

To elicit laccase function virus-induced gene silencing (VIGS) was applied in *Nicotiana benthamiana* plants. The VIGS vectors are obtained by manipulation of the viral genome to smooth inserting host DNA sequence to the corresponding host RNAs. Then the transformed vectors with interesting gene are introduced to the plant and targeted by plant defense system. In VIGS system, the endogenous transcripts of plant gene that are homologous to the inserted sequence in the viral vector are erroneously recognized to be degraded by plant defense system (Baulcombe, 1999 and 2004).

The vectors derived from tobacco rattle virus (TRV) are widely used as VIGS vectors in various plants, especially in *Solanaceae* family members (Ratcliff *et al.*, 2001; Brigneti *et al.*, 2004; Dong *et al.*, 2007). Some other vectors including tobacco mosaic virus (TMV), tomato mosaic virus (ToMV) (Kumagai *et al.*, 1995), tomato golden mosaic virus (TGMV) (Kjemtrup *et al.*, 1998) and potato virus X (PVX) (Ruiz *et al.*, 1998) were

used as VIGS vectors. These vectors cause common symptoms of leaf chlorosis and leaf distortion, while the TRV vector induced a mild symptom that was not confused with VIGS phenotype (Ratcliff *et al.*, 2001). On the other hand, TMV and PVX are unable to infect the apical meristem, therefore they are not suitable to be used in the studies of genes involved in development of plant tissues and organs (Chen *et al.*, 2004). In contrast to PVX, the TRV virus infected systematically wide tissue area and passed through the growing points resulting in silencing expression of genes in the meristem (Ratcliff *et al.*, 2001).

Laccase is a copper containing glycoprotein and many functions have been attributed to laccase enzymes in higher plants. Involvement of laccase in lignin biosynthesis and degradation, pigment formation, detoxification and pathogen defense response was reported (Solomon *et al.*, 1996; Mayer and Staples, 2002; McCaig *et al.*, 2005). Sato *et al.* (2001) hypothesized that the predominant expression of laccase in differentiating xylem of pine may play role in xylem development by lignin synthesis. Some laccase-like genes expressed differentially in *Theobroma cacao* (cocoa) was identified. These peroxidase genes showed to be involved in resistance of cocoa to witches' broom disease caused by *Crinipellis perniciosa* (Leal *et al.*, 2007). Expression of laccase by ethylene (Ahmadi *et al.*, 2008) should be considered, especially when this gene was expressed in some plants by abiotic stress (Liang *et al.*, 2006), salinity and abscisic acid (Wei *et al.*, 2000). Expression of laccase-like multicopper oxidase in young roots, containing no more lignin, shows that the products of this enzyme play some roles in physiological functions as well as lignin deposition (McCaig *et al.*, 2005). Although the laccase enzyme plays roles in lignin biosynthesis and degradation (Nakamura, 1967) but increasing expression under some biotic and abiotic stresses in herbaceous plants may be related to plant stress response. Using mutants for laccases in *Arabidopsis*, some physiological functions attributed to laccases like root elongation, earlier flowering and changing seed color (Cai *et al.*, 2006).

Based on previous studies, we hypothesize that laccase may be functionally involved in specific response to stressful conditions induced by ethylene (Ahmadi *et al.*, 2008) as well as abscisic acid, salinity (Wei *et al.*, 2000) and some abiotic stress factors (Liang *et al.*, 2006). The main goal of this study is to evaluate the functional and physiological role of laccase gene in plants exposed to exogenous ethylene.

Materials and Methods

Plant materials

Nicotiana benthamiana seeds were germinated in multiwell plates (one seed per well). The seeds were germinated at 23-25 ^{o}C with a 16 h (light intensity 350 µmol/m^2/s) to 8 h light/dark regime. Two weeks after germination, plugs were transplanted in 10 cm-diameter pot. The 26-day old plant with3-4 expanded leaves were used for *Agrobacterium tumefaciens* inoculation.

Cloning of portions of the *RhLAC* and β-glucuronidase genes into the pTRV2vector

The partial sequence of rose laccase (*RhLAC*, 560 bp, Accession No. EU603403) was amplified using PCR primers, forward 5′-GAACCACCCCATTGATGTTC-3′ and reverse 5′-GATGGCAGTGCATAAACCAA-3′. As nonsense cDNA, β-glucuronidase gene (798 bp) from binary vector pBI121 of *Escherichia coli* (Accession No. AF485783) was amplified using PCR primers, forward 5′-TTTTTGTCACGCGCTATCAG-3′ and reverse 5′-CAACGAACTGAACTGGCAGA-3′. This nonsense (NS) fragment does not exist in plant genome to be silenced and only used as control cDNA. Amplified fragments were cloned separately in pGEM®-T Easy vector according to company instruction. To prepare *EcoRI* site, the transformed pGEM®-T Easy vectors containing target cDNA as insertions were cut with *EcoRI* enzyme to be used in the subsequent plasmid construction. The pTRV2 plasmid vector was linearized by

overnight incubation with *EcoRI* enzyme at 37 °C.
The target cDNAs (*LAC* or NS) were cloned into the *EcoRI* sites of the pTRV2 plasmid vector according to the following procedure. Totally 20 µl of reaction mixture containing 45 ng corresponding cDNA, 15 ng pTRV2 vector, 3 unit T4 DNA ligases enzyme (Promega Co.) and 5 µl 10X ligation buffer incubated at 4 °C overnight. Transformation was carried out by adding 2.5 µl of ligation reaction to 50 µl of *E.coli* competent cells. Positive transformed bacteria were selected by PCR reaction and targeted clones were grown liquid LB (50 ng l^{-1} Kanamycin) at 37 °C overnight and plasmid DNA was isolated by using the NucleoSpinR Plasmid Kit (Macherey-Nagel Co.).
Agrobacterium tumefaciens strains GV3100 and GV3101 were transformed by pTRV1 and pTRV2 vectors respectively, using MicroPulser™ (BioRad). The cells were then transferred to a 250 µl S.O.C medium and incubated for 3 hours at 28 °C and plated on LB medium containing 25 mg/l Rifampicin and 50 mg/l Kanamycin for selection of transformants. The transformed colonies appeared on the selection medium after 48 hours of incubation at 28 °C.
The *A. tumefaciens* transformed by pTRV2 vector carrying partial phytoene desaturase (*PDS*) gene was kindly provided by Dr. Merete Albrechtsen (University of Århus, Denmark).

Viral inoculation of plants

Selecting the positive transformed *A. tumefaciens* clones, bacteria were cultured to an OD_{600} of 0.6-0.7. The cultures were centrifuged at 5000 rpm for 15 min at 4 °C and the pellet was dissolved with infiltration buffer (10 mM MES and 10 mM MgCl2, pH, 5.6) to an OD_{600} of 1. To enhance the transformation efficiency, acetosyringone (150 µM) was added to suspension (Joao and Brown, 1993) followed by gently shaking at room temperature for overnight. *A. tumefaciens* carrying pTRV1 or pTRV2 were mixed in ratio of 1:1. Using needleless syringe, the prepared agro-inoculations were infiltrated into the lower surface of three to four leaves of *N. benthamiana* plants. In each replication, 10 plants were inoculated with *A. tumefaciens* carrying *LAC* and NS fragments. After 5 days, the plants were placed in two identical aquarium glasses, exposed to 0 or 5µl/l injected ethylene. Ethylene concentration was monitored daily using a Perkin-Elmer portable digital gas chromatograph (GC Voyager FFKG312) equipped with a photoionisation detector. Oven and column temperatures were set on 60 °C, injection pressure was 69 kPa and the N_2 carrier gas flow was 40 ml min^{-1}.
For evaluation of VIGS efficiency, 5 plants inoculated with *A. tumefaciens* carrying *PDS* were put out side of aquarium glasses to monitor photobleaching.

Evaluation of plant morphological characteristics

After 15 days of ethylene treatment, the number of dead, yellow and green leaves was counted. The plant height was measured with a 1 mm-graduated ruler. The distance extended from crown to apical meristem was considered as the height of the plant. The total green leaves area was measured using ImageJ software, a Java-based image processing program developed at the National Institutes of Health (http://rsb.info.nih.gov/ij/).

Results

To gain insight into laccase function in plants, the plant seedlings of *N. benthamiana* were infiltrated with two strains of transformed *A. tumefaciens* carrying TRV2+laccase (*LAC*) fragment and TRV2+nonsense cDNA sequence as control (NS). 5 days after inoculation, the infected plants were treated with 5 and 0 µl/l exogenous ethylene.
The most prominent effect of ethylene and silencing laccase gene on plant morphological characteristics appeared in plant growth and development. The height of the plants was significantly decreased by laccase gene silencing and ethylene treatment. Ethylene reduced significantly the size of laccase silenced plants (Fig. 1 and 2). Ethylene treatment decreased the total number of leaf

(Fig 3). The higher number of yellow leaves appeared in non ethylene treated *LAC* silenced plant. The number of dead leaves was increased by ethylene treatment. In non-ethylene treatment, laccase gene silenced plants showed higher number of dead leaves than NS plants. Non-ethylene treated NS plants showed significantly higher number of green leaves and the lower number of yellow leaves. The total green leaves area was reduced by *LAC* silencing gene and ethylene treatment. The lowest level of green leaves area showed in ethylene treated *LAC* silenced plants (Fig 4). The old leaves were paled and finally dead while attached to the stem. Neither ethylene treatment nor *LAC* gene silencing caused leaf abscission in *N. benthamiana.*

Discussion

To evaluate the function of the previously cloned *RhLAC* (Ahmadi *et al.,* 2008), the virus induced gene silencing (VIGS) method was used to suppress posttranscriptional expression in *N. benthamiana* (Baulcombe, 1999). This technique was used successfully in the study of gene function in tomato (Xie *et al.*, 2006), and hot pepper (Kim *et al.*, 2007). Considering the advantages of TRV vector, it was used as vector in this experiment. As control infection, plants were inoculated with pTRV2 carrying 798 bp fragment of glucuronidase gene derived from bacteria as nonsense (NS) fragment. This exogenous gene fragment is not causing any changes in plant biochemical pathways.

In this study, the phytoene desaturase (*PDS*) reporter gene was applied to estimate the feasibility and efficiency of gene silencing process. Inoculation of plant with TRV+ *PDS* decreases the transcript abundance of *PDS* resulting in distinctive leaf photobleaching. Phytoene desaturase plays role in carotene formation pathway (Chen *et al.*, 2004).

Our previous experiment showed that the photobleaching appeared after 6 to 7 days of inoculation. We supposed that the silencing process initiated earlier than the 7 day period time and the whitened symptom emerged on new leaves coming out after 6 to 7 days. Base on this assumption, the applying of exogenous ethylene was carried out 5 days after infiltration.

The main effect of *RhLAC* gene silencing was revealed as stunting in plant growth, comparing to control (NS) (Fig. 1). *LAC* silencing caused to decrease the plant size and leaves area. Ethylene treatment promoted the negative effects caused by laccase silencing (Fig. 2 and 4).

Reduction of plant growth may be directly related to the cell growth process that was disorderly affected by laccase silencing or ethylene. The involvement of ethylene in the regulation of root and hypocotyls growth was frequently reported with the wealth of information on this subject. Analysis of triple response during ethylene treatment or in mutants carrying mutated genes of ethylene biosynthesis or perception pathways has showed that ethylene can negatively regulate the elongation growth in *Arabidopsis* (Kieber *et al.*, 1993; Roman and Ecker, 1995).

In this study, silencing of *LAC* gene affects growth and development of *N. benthamiana.* To date 17 laccase genes were isolated from *Arabidopsis thaliana* (Caparros-Ruiz *et al.*, 2006; McCaig *et al.*, 2005). Several reasons make difficult to interpret the results of laccase silencing in *N. benthamiana.* At first northern blot analysis should be done to confirm occurring gene silencing. It may be some other silenced genes that showed homology to target fragment. Blast analysis of inserted laccase nucleotide sequence showed 61% identity to laccase 15 in *Arabidopsis thaliana.* Laccase 15 involved in oxidative polymerization of flavonoids in the seed coat (Pourcel *et al.*, 2005; Cai *et al.*, 2006), lignin synthesis and root elongation (Liang *et al.*, 2006). Since the experimental course time was terminated before flowering, it was not possible to find the laccase role in early flowering and seed color (Cai *et al.*, 2006).

Base on available data, we postulate that laccase enzyme may somehow be involved in cell division and/or cell enlargement. This idea is boosted by the findings of laccase activity in regenerating protoplasts tobacco and suggestion of laccase involvement in cell wall formation without participation in lignification (De Marco and Roubelakis-

Angelakis, 1997). In another study, laccase gene (*LpLAC*2-1) expression was at high level in meristem and stem, and at low level in roots of ryegrass (Gavnholta *et al.*, 2002). Moreover, four kinds of laccase mRNAs were accumulated in root elongation zone in maize, (Caparros-Ruiz *et al.*, 2006). These findings support that laccase may participate in cell division and/or cell elongation.

Our previous report pointed out the induction of laccase gene expression under ethylene treatment (Ahmadi *et al.*, 2008). In this study ethylene has retarded the plant growth and leave extension, and its retarding effects were strongly severe on *LAC* silenced gene plants. Laccase expression increased under ethylene treatment, biotic and abiotic stress conditions (Liang *et al.*, 2006; Wei *et al.*, 2000). It may be due to encountering the plant to stressful conditions and the expression of laccase protects the plant from these deteriorative situations.

Yellowing of older leaves leads to dead of these leaves, especially under ethylene treatment. *Nicotiana* sp. plants do not abscise leaves in reaction to ethylene treatment, although other parts such as floral buds and petals collapse (Aharoni, 1978; Yang *et al.*, 2008). This was unfortunately a weak point in using this plant in study of leaf abscission process.

In conclusion, silencing the laccase gene retarded growth and development of *N. benthamiana* by reducing height of plant and total green leaf area. Similarly, ethylene suppressed growth rate and induced leaf yellowing. The effect of ethylene treatment was more distinguished in the laccase silenced plants than in control. Silencing of laccase gene may suppress cell division and/or cell enlargement. Due to variation of laccase enzymes and controversial physiological functions ascribed to laccase, it is not easy to interpret precisely these results. Therefore more studies are necessary to shed light on the physiological function of laccase genes.

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Ethylene Non ethylene

Fig 1. The laccase gene silenced (above) and control (NS) *N. benthamiana* plants exposed to ethylene (left) and non ethylene treatment (right). The plants (1.5- 2 cm height) were treated with ethylene (5 µl/l) and non ethylene for 12-day period.

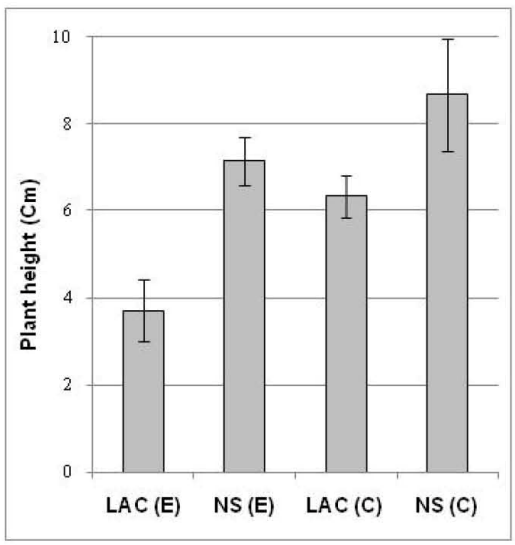

Fig 2. The height of laccase gene silenced (*LAC*) and control (NS) *N. benthamiana* plants exposed to ethylene (E) or ethylene-free air(C). The 1.5- 2 cm height plants were treated with 0 or 5 µl/l ethylene for 12-days.

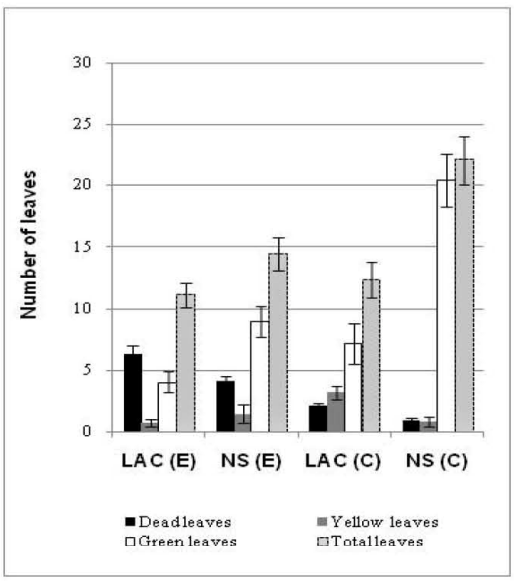

Fig 3. The number of dead, yellow and green leaves of laccase silenced (*LAC*) and control (NS) *N. benthamiana* plants exposed to ethylene (E) or ethylene-free air (C). The plants containing three to five leaves were treated with 0 or 5 µl/l ethylene for 12-days.

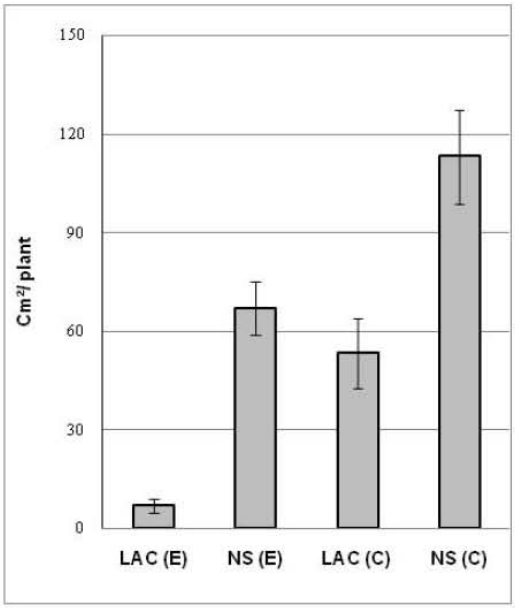

Fig 4. Total green leaves area of laccase silenced (LAC) and control (NS) *N. benthamiana* plants exposed to ethylene (E) or ethylene-free air (C). The plants containing three to five leaves were treated with 0 or 5 µl/l ethylene for 12-days.

Identification of ethylene induced genes in abscission zone of *Rosa hybrida* L. by use of differential display

Noorollah Ahmadi, Heiko Mibus and Margrethe Serek*

Faculty of Natural Sciences, Institute for Ornamental and Woody Plant Science, Leibniz University of Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany; *Author for correspondence (e-mail: serek@zier.uni-hannover.de; phone: +49-511-7622657; fax: +49-511-7622654)

Keywords: abscission, differential display, ethylene, *Rosa hybrida*

Abstract

Ethylene plays a crucial role as a coordinator of floral senescence in many ornamental plants, especially during handling and shipping. The most deteriorating effects of ethylene on miniature rose quality appear as leaf yellowing and abscission, petal wilting or flower abscission. Ethylene has a regulatory effect at the transcriptional and translational levels and the study of the molecular aspects of these regulations shed light on the ageing process.

The pedicel of miniature roses, *Rosa hybrida* L. cv. turns yellow after exposure to ethylene. RNA was isolated from pedicels and petioles which were exposed to exogenous ethylene for 0 h or 72 h. In order to identify ethylene induced genes, Differential Display Reverse Transcription Polymerase Chain Reaction (DDRT-PCR) was carried out on mRNA isolated from pedicel and petiole of ethylene treated and non treated rose plants. A total of 88 cDNA fragments were found to be up-regulated, whereas 72 were down-regulated in response to ethylene. Five ethylene-response cDNAs out of 88 were confirmed as differentially expressed by qRT-PCR. Three of them were identified from petiole and two from pedicel. The differential expression level of these cDNAs was assayed in various tissues of non- and ethylene treated miniature roses.

Introduction

Plant hormone ethylene, produced naturally in plants, at trace amount regulates many physiological and developmental processes from seed germination to senescence (Abeles, 1992). Ethylene plays important role in the display quality of ornamental plants and most of research work has been focused on the ethylene biosynthesis and its action in plants. The effects of endogenous or exogenous ethylene treatment on miniature potted rose appear as leaf yellowing, flower senescence, leaf and floral bud abscission. These detrimental effects of ethylene reduce the growers' income (Serek and Reid 1994; Müller *et al*., 1998). The response of miniature potted rose plants to ethylene varies among the cultivars (Buanong *et al*., 2005; Müller *et al*., 2001).

Recently it was reported that the floral organ abscission in *Arabidopsis* are dependent and also independent to ethylene action. Ethylene treatment of delayed floral organ abscission mutants accelerates leaf senescence and abscission. Patterson and Bleecker (2004) suggest that sensitivity of these mutants at the seedling and maturity stages to ethylene is an important observation in recognizing abscission can be regulated independently of ethylene.

The organ abscission phenomenon caused by catabolic activity in the cell wall of the abscission zone is being related to activities of two gene families, cellulases (endo1, 4-ß-D glucanase) and polygalacturonases. Other enzymes have been assigned to be involved in abscission process including chitinases, ß-1,3-glucanase, uronic acid oxidase and peroxidases, (Sexton et al., 1985; Brown, 1997).

It is well known that ethylene has a regulatory effect at transcriptional or translational level of genes involved in cell separation (Brown 1997). The ethylene biosynthesis pathway has been documented in numerous studies, but in the case of ethylene perception and signal transduction and transcriptional pathway, more controversial topic in this context must be discussed. In this paper, we applied Differentially Display Reverse Transcription Polymerase Chain Reaction (DDRT-PCR) innovated by Liang and Pardee (1992) to isolate differentially expressed mRNA under ethylene treatment.

MATERIALS AND METHODS

Plant materials and ethylene treatment

The tetraploid miniature rose (*Rosa hybrida* L. cv. Lavender) plants from Kordana breeding line (W. Kordes' Söhne Rosenschulen GmbH & Co KG, Germany) were used in this study. The plants were propagated and grown 3 cuttings per pot in the greenhouse at Leibniz University of Hannover. The growing conditions were 20 °C/20 °C (day/night) and 70% relative humidity (RH). Day length was extended to 16 h by SON-T lamps (Osram, 400W, Philips Co.) supplied 260 µmol m^{-2} s^{-1}.

Plants were placed in 200 l glass chambers. The chambers were ventilated with air carrying ethylene 1.5 (±0.25) µl l^{-1} for 72 h. Control plants were placed in identical glass chambers under the same conditions, but without ethylene. Ethylene concentration was daily monitored using a Perkin-Elmer portable digital gas chromatograph (GC Voyager FFKG312). Samples of bud pedicels, leaf petioles, leaf abscission zones (LANZ), bud abscission zones (BANZ), leaf blades and petals were collected from 80 potted plants. The LANZ sample consisted of stem-petiole abscission zone and 2 mm of basal petiole attached to the stem containing a stipule; BANZ sample consisted of floral bud abscission zone with 2 mm of the basal part of the floral bud. Plant samples were frozen immediately in liquid nitrogen, ground with a mortar and pestle in liquid nitrogen and stored at –80 °C until extraction of RNA.

Extraction of RNA and Reverse Transcription

Total RNA was extracted from 40-60 mg of ground plant samples using Invisorb^{R} Spin Plant RNA Mini Kit (Invitek Co.). In order to remove chromosomal DNA contamination, each 2 µg RNA sample was treated with 0.5 U of *DNase* I (Fermentas Co.), 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 20 U RiboLock^{TM} (Fermentas Co.) in 10 µl total volume and incubated at 37 for 30 min. *DNase* residual was removed by adding 2 mM EDTA followed by incubation at 65 °C for 10 min.

For reverse transcription 0.5 µg of DNA-free total RNA was initial incubated with 2.5 µM anchor primer (D1- D12, Biometra, Co.) at 70 °C for 7 min and quenching immediately on ice for 5 min. After a brief centrifugation, 10 mM Tris-HCl, 15 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM DTT, 0.5 mM dNTPs, 200 U M-MLV RT (H-) enzyme, 20 U RiboLock^{TM} (Fermentas Co.) were added to the 12.5 µl volume of RT reaction and incubated at 40 °C for the first 10 min, 50 °C for 150 min and finally 70 °C for 5 min.

Differential display – PCR

PCR amplification was carried out in a 20 µl reaction mixture containing 10 ng of cDNA template, 150 µM each of dNTP, 2.5 µM downstream primer (corresponding to reverse transcription), 0.5 µM upstream random primer (U1-U26), 0.5 U Taq DNA polymerase (Invitek Co.), 10 mM Tris-HCl, 50 mM KCl, 2mM MgCl2. PCR amplification was conducted in the Thermocycler (Biometra Co.) under the following conditions: 30 s at 94 °C for initial denaturation, followed by 46 cycles consisting of 30 s at 94 °C for denaturation, 1 min at 40 for annealing, 1.5 min at 72 °C for polymerase extension and a final extension step of 72 °C for 10 min.

PCR reaction products were separated by 1% agarose gel stained with ethidium bromide and the sizes of amplicons were evaluated by comparing them to a 100 bp DNA marker (Fernentase Co.). The amplified polymorphic bands occurring as a result of ethylene treatment were cut from the gel and stored at 4°C. For cloning, the cDNA fragments were purified from agarose gels using the Invisorb® Fragment CleanUp kit (Invitek Co.).

Cloning and sequencing of polymorphic fragments

The cDNAs up-regulated under ethylene treatment were ligated into TA plasmid vector using pCR^{R}4-TOPO^{R} TA Kit (Invitrogen Corp.). Positive transformed bacteria were identified by PCR. Plasmid DNA from transformed *E. coli* was isolated using the NucleoSpinR Plasmid Kit (Macherey-Nagel Co.) and sequenced by MWG Biotech Co. The isolated sequences were analysed using the CLUSTAL W (EMBL; Higgins 1994) and BLUSTN programs (NCBI; Altschul et al., 1997).

Real-time RT-PCR Assay

To evaluate expression patterns of isolated cDNAs, reverse transcription reactions were carried out using 1µg oligo dT(23) primer as described previously. The PCR reaction mixture was made up to a volume of 20 µl containing 10 ng of cDNA template, 150 µM of each dNTP, 2.5 µM specific forward primer, 2.5 µM specific reverse primer, 1 U Hot Start Tag DNA polymerase (Axon Co.), 5 mM $MgCl_2$, 10 mM Tris-HCl and 50 mM KCl. SYBR-Green (Roche Applied Science, Co.) added to the reaction mixture was used to monitor the amplification of PCR products. Incubating 10 min at 94 °C, the cDNA was amplified by 45 three-step cycles: 30 s at 94 °C, 1.5 min at 62 °C and 2 min at 72 °C. The expression levels of ß–actin were detected for each sample concomitantly with specific genes to normalize all samples using Rotor Gene software (version 6.1.81). The relative quantification of transcript abundance of target genes was determined by the $2^{-\Delta\Delta CT}$ method in individual plant samples (Livak and Schmittgen 2001).

RESULTS

Applying 12 arbitrary primers in combination with 26 anchoring primers, 1910 fragments were detected varying from 100 to 1200 bp. 88 up-regulated and 72 down-regulated fragments were isolated in response to ethylene. Finally 17 cDNA fragments out of 88 expressed differentially following ethylene treatment were cloned and sequenced. Seven cDNA bands did not show any significant similarity to sequences existing in the database and five bands showed similarity to bacterial, fungal and human genom database. Totally five cDNAs were identified, two from bud pedicels (cDNA-3 and 4) and three from leaf petioles (cDNA-1, 2 and 5).

Table 1 shows the isolated cDNAs homologos searched in database. The cDNA-1 showed high homology to *Cucumis melo* UDP-galactose/glucose pyrophosphorylase. The sequence of cDNA-2 and cDNA-3 are homologous to *Arabidopsis thaliana* HB-1 (homeobox-1) and *Cypripedium parviflorum* trehalose-6-phosphate synthase/phosphatase respectively. The homologues of cDNA-4 are found in *Solanum demissum* and *Arabidopsis thaliana* for kelch repeat-containing F-box family protein. cDNA-5 was approximately 77% homologous with putative laccase in *Zea mays* (accession CAJ30499) and 51% homologous with laccase 14 (accession NP_196498) from *Arabidopsis thaliana.* All five isolated cDNAs were expressed at least 2-fold in pedicel. In petiole all cDNAs showed less than 2-fold expression, except cDNA-5 that expression was about 9-fold compared to control (Table 2). The partial cDNA-5 is henceforth termed *Rosa hybrida* Laccase gene (*RhLAC*), whose expression was higher than others applied for further study. The highest levels of *RhLAC* expression showed in the leaf abscission zone (LANZ) and bud abscission zone (BANZ) respectively. The relative expression of *RhLAC* was lowest level in leaf blades, and it is highly expressed in petal and pedicel samples (Table 3).

DISCUSSION

Using the differential display method, five partial cDNA transcripts were isolated from pedicel and petiole of rose treated with ethylene. About 80% of differential display amplicons appeared falsely in this experiment and this DDRT-PCR disadvantage was previously discussed (Debouck, 1995).

The cDNA-1 and cDNA-2 showed high homology to *Cucumis melo* UDP-galactose/glucose pyrophosphorylase and *Arabidopsis thaliana* HB-1 (homeobox-1) respectively. The sequence of cDNA-3 is homologue to gene encoded trehalose. Trehalose with stress protection charactristics is accumulated in cells under stress conditions (Van Dijck and others 2002). The cDNA-4 homologue is kelch repeat-containing F-box family protein founded in *Solanum demissum* and *Arabidopsis thaliana.* Probably the F-box protein plays role to specify proteins for degradation in programmed cell death. The analysis of cDNA-5 showed 77% identity to putative laccase in *Zea mays*. This unknown cDNA-5 is assumed to be a putative lacctase in *Rosa hybrida* and so designated as *RhLAC* gene

Relative expression of *RhLAC* gene in leaf blade and petiole was lower than other tissues. It might be related to ethylene production endogenously in non-treated corresponding samples. In a separate study of non-ethylene treated leaf blade and petiole of plants grown in glass chambers and greenhouse, the *RhLAC* was expressed at low level in plant placed in glass chamber but was not expressed in greenhouse plants. It could be resulted from some stressful conditions in glass chamber such as high humidity (data not shown).

Laccase is a multi-copper glycoprotein enzyme containing four copper atoms per protein molecule. It participates in many physiological functions including lignin biosynthesis, lignin degradation, wound-healing process, pigment formation and production of antimicrobial compounds (Mayer and Staples 2002, McCaig *et al*., 2005). Similar to the effect of ethylene treatment, the expression of laccase gene was raised by abscissic acid and salinity treatments in other plants (Wei et al., 2000; Liang et al., 2006).

Regardless of much research work, genetic evidence for the role of laccase in plants is not so clear. Here we propose that the laccase might play role in wound-healing process after organ abscission. The detachment of organs from the main body of the plant leads to cause open wounds on the abscission zone and laccase may be involved in repairing of damaged parts by regeneration of protoplasts and lignin deposition in the wounded regions (Mayer and Staples 2002).

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Tables

Table 1. List of isolated ethylene up-regulated cDNA sequences, their size, e-value and homology to the sequences available at the GenBank.

cDNAs	Size (bp)	Homology to (Accession number)	e-value
cDNA-1	706	ABD59006	8e-40
cDNA-2	437	NP_174164	2e-25
cDNA-3	1064	AAN86570	2e-31
cDNA-4	629	AAT40540	2e-90
cDNA-5	447	CAJ30499	1e-08

Table 2. Relative expression of the isolated cDNAs genes induced in pedicel and petiole of rose after 72 h ethylene treatment. Symbol (±) represents the standard deviation.

cDNAs	Pedicel	Petiole
cDNA-1	3.59 (± 0.3)	1.05 (± 0.1)
cDNA-2	2.48 (± 0.3)	1.79 (± 0.4)
cDNA-3	6.92 (± 1.0)	1.62 (±0.3)
cDNA-4	15.33 (± 1.7)	1.35 (±0.4)
cDNA-5	54.16 (±1,5)	9.70 (± 2.5)

Table 3. Relative expression of the *RhLAC* gene expressed under 72 h ethylene treatment in different organs and tissues. Symbol (±) represents the standard deviation.

Tissues and organs	Bud abscission zone (BANZ)	Leaf abscission zone (LANZ)	Pedicel	Petal	Petiole	Leaf blade
RhLAC expression	90.04 (±6.1)	106.25 (± 4.8)	54.16 (±5.4)	80.89(±5.0)	9.7 (±2.5)	3.02 (±1.2)

Leibniz Universität Hannover

Isolation of Ethylene Induced Putative Nucleotide Laccase in Miniature Roses (*Rosa hybrida* L.)

Noorollah Ahmadi, Heiko Mibus and Margrethe Serek

Faculty of Natural Sciences, Institute for Ornamental and Woody Plant Science, Leibniz University of Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany

Introduction

Ethylene as plant growth regulator accelerates leaf and floral bud abscission and flower senescence in potted rose plants (Serek and Reid 1994). Objective of the present study was to isolate genes expressed from abscission zone of pedicel and petiole, following ethylene treatment by employing differential display RT-PCR (Liang and Pardee 1992).

Material and Methods

Differential Display- Reverse Transcription PCR was carried out using purified total RNA extracted from 72 h ethylene (1,5 ppm) treated bud pedicels and leaf petioles and from control samples of the potted rose `Lavender` (W. Kordes GmbH & Co KG) . To quantify mRNA levels, real-time RT–PCR assays were performed using Rotor Gene 3000. The relative quantification of transcript abundance of target genes in individual plant samples was determined by $2^{-\Delta\Delta CT}$.

Results and Discussion

Table 1. List of isolated ethylene up-regulated cDNA sequences.

cDNAs	size (bp)	Homology (Acc. No.)	e-value
cDNA-1	706	ABD59006	8e-40
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cDNA-5	447	CAJ30499	1e-08

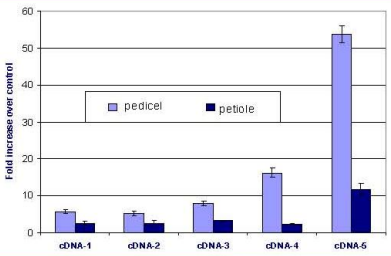

Figure 1. Relative expression of the isolated cDNAs following 72h ethylene treatment after normalization to the ß–actin gene. Vertical bars represent ± standard deviations (n=3).

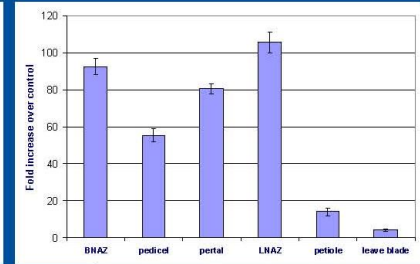

Figure 2. Relative expression of the *RhLAC* gene expressed under 72 h ethylene treatment in the bud abscission zone of floral bud pedicel (BANZ), pedicel, petal, leaf abscission of petiolar pulvinus (LANZ), petiole and leaf blade after normalization to the ß–actin gene. Vertical bars represent ± standard deviations (n=3).

Five cDNA fragments were isolated (Tab. 1 and Fig. 1). The full-length of cDNA-5 assigned as *RhLAC* was 2005 bp that encoded a 63 kDa polypeptide (573 amino acids). Three putative conserved domains of the multi-copper oxidase family were detected in the amino acid sequences levels of this gene. The analysis of the deduced amino acid sequence indicated a 58% identity to putative laccase in *Zea mays* and 56% to laccase 15 in *Arabidopsis*. Comparison of the relative expression of isolated *RhLAC* in various organs showed that it was highly induced in the leaf abscission zone of petioles and the bud abscission zone of floral bud pedicels, while it was low in both leaf blades and petioles (Fig. 2). Laccase plays role in lignin biosynthesis and degradation, wound-healing and pigment formation (Mayer et al., 2002). Since the transcrption level of laccase also increased following stressful conditions (Liang et al., 2006), this gene might be involved in response to stress condition caused by ethylene and /or in wound-healing processes occurring after organ abscission.

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Discussion and Outlook

4. Discussion and Outlook

The goal of this basic research was to gain increased understanding of physiological and molecular aspects of miniature roses reaction to ethylene. This may lead to development of a selection mechanism to characterize ethylene sensitivity of rose plant in breeding program of potted rose. At first step, a population of two F1 breeding lines was screened to evaluate sensitivity of genotypes to ethylene. Synchronously, the Differential-Display Reverse Transcription PCR (DDRT-PCR) was performed to identify unknown genes expressed under ethylene treatment. The physiological response of selective genotypes and their parents were evaluated in terms of leaf yellowing and chlorophyll content. At molecular level, the relative expression of ethylene receptors, genes involving in transduction pathway and laccase genes were studied in ethylene-treated and non-treated pedicels and petioles of selective genotypes and their parents.

4.1 Evaluation of leaves and buds abscission induced by exogenous ethylene

In breeding program of miniature roses, breeders mainly focus on the visible traits such as flower color and shape in selection of new cultivars, however, information about postharvest characteristics of offspring is low. In this study, extensive pool genes prepared by reciprocal crossing of two miniature rose cultivars, ‘Vanilla’ and ‘Lavender’ resulted in obtaining of two F1 generations. The cultivar ‘Vanilla’ is known as low sensitive (Müller *et al.*, 1998) and ‘Lavender’ has been considered as high sensitive to exogenous ethylene (Buanong *et al.*, 2005).

To select the highest and lowest sensitive genotypes of potted rose plants to exogenous ethylene, 232 progenies of two F1 generations were screened in term of leaf abscission rate. The average of leaf numbers varied approximately from 120 to 190 leaves per pot of genotypes and the moderate number of buds was 6-12. Due to plenty of leaves and few floral buds, leaf abscission was considered for evaluation in ethylene sensitivity instead of bud abscission.

All of the genotypes investigated showed different reaction to exogenous ethylene, ranging from less than 20% to more than 80% leaf abscission after 5 days of ethylene

treatment **(Fig. 1, manuscript 1)**. Selection of the low sensitive genotypes from both F1 generations showed that 'Vanilla' could serve as a potential genetic resource for breeder to improve ethylene-related display quality in miniature rose **(Fig. 2 and 3, manuscript 1)**. Considering distribution of genotypes in term of leaf abscission rate after 5 days ethylene treatment showed that using 'Vanilla' in crossing as mother or father did not affect transferring traits related to improving display quality. In 'Lavender' less than 10% leaf abscission exhibited after 3 days and about 80% on day 6 of ethylene treatment. These findings agree with Buanong *et al*. (2005). 'Lavender' defoliated all leaves after 9 day of ethylene treatment in both studies (**Fig. 2, manuscript 1**; Buanong *et al*., 2005).

In this study, less than 40% 'Vanilla' leaves were abscised after 5 day ethylene treatment **(Fig. 2, manuscript 1)**. Müller *et al*. (2001a) reported that 'Vanilla' abscised less than 40% leaves after 6 days exposure to 5 $\mu l\ l^{-1}$ ethylene. In this study 20% leaves were dropped by 0.5 $\mu l\ l^{-1}$ ethylene treatment. This difference could be related to the threshold of ethylene concentration for initiation of leaf abscission. Increasing ethylene concentration from 1.5 to 5 $\mu l\ l^{-1}$ may not change the leaf abscission rate in this cultivar. In this study, about 20% buds of 'Vanilla' were dropped after 5 days of 1.5 $\mu l\ l^{-1}$ ethylene treatment after 6 day **(Fig. 3, manuscript 1)**. About 5% and 40% of bud abscission occurred by 0.8 and 8 $\mu l\ l^{-1}$ ethylene respectively (Müller *et al*., 1998).

4.2 Leaves yellowing and chlorophyll degradation

Selected high and low sensitive genotypes and their parents, 'Vanilla' and 'Lavender' were evaluated for yellowing and senescing symptoms. Generally, ethylene application increased chroma values in all genotypes and parents. Yellowing symptom was detectable in high sensitive genotypes after 7 days ethylene treatment, but it developed slowly in low sensitive genotypes **(Fig. 4 and 5, manuscript 1)**. Ethylene accelerated leaf yellowing by chlorophyll degradation. This is consistent with the observation that leaf yellowing was strongly accelerated by exposing miniature potted rose plants to ethylene (Tjosvold *et al*., 1995; Serek *et al*., 1996). This result is in agreement with application of 1-MCP, an ethylene action inhibitor that prevented degradation of chlorophyll and leaf yellowing in miniature potted rose (Hassan *et al*., 2004). In cut rose

cv.'Saphir', the extracted chlorophyll amount was higher after 1-MCP treatment, while it was not changed in cv.'Confetti' (Cuquel *et al*., 2007). Ethylene treatment accelerated yellowing in *Nicotiana benthamiana*. This yellowing symptom leads to death of leaves attached to plant without any abscission process **(Fig. 3, manuscript 2)**. Not only the exogenous ethylene but also endogenous ethylene accelerated yellowing in attached leaves of tobacco cultivars (Alejar *et al*., 1988). In citrus peel, ethylene was involved in chlorophyll degradation and removing ethylene ceased chlorophyll degradation (Purvis and Barmore, 1981). The effect of ethylene on chlorophyll has been intensively studied on various types of vegetables. Ethylene enhances chlorophyllase activity and extends the period of high peroxidase activity in broccoli (Gong and Mattheis, 2003). Chlorophyllase cleaves chlorophyll into phytol and chlide, the Mg- porphyrin moiety of chlorophyll (Matile *et al*., 1996; Rodoni *et al*., 1997). The chroma value and chlorophyll content of low sensitive genotypes did not change significantly during 15 days ethylene treatment **(Fig. 4 and 5, manuscript 1)**. Hence, the deleterious effects of ethylene on leaf yellowing and chlorophyll degradation could be related to genotype. These data agree with observations on miniature rose plants (Serek *et al*., 1996) and cut rose (Cuquel *et al*., 2007) that ethylene accelerates yellowing but is not primary agent (Serek *et al*., 1996). Considering our result that leaves of laccase homologue silenced plants became yellow and died in ethylene-free air **(Fig. 3, manuscript 2)**, there could be no direct relationship between ethylene and yellowing.

4.3 Expression of *RhETR1*, *RhETR3*, *RhCTR1* and *RhCTR2*

Müller *et al*. (2000b) reported that differences in flower life among miniature potted rose cultivars could be due to differences in expression of receptors levels. Thus, the relative expression of *RhETR1* and *RhETR3* under ethylene treatment was evaluated in selected genotypes and their parents, 'Lavender' and 'Vanilla'. Accumulation of *RhETR1* transcript in pedicel and petiole of long-lasting 76/67 genotype **(Fig. 6 and 7, manuscript 1)** is in agreement with findings in cut rose cv. Samantha. Transcript levels of *RhETR1* and *RhETR3* were elevated in petals of cv. Samantha and ethylene caused flower opening in this cultivar (Ma *et al*., 2006). While in cv. Kardinal, the *RhETRs* expression did not induced by ethylene and resulted in inhibition of floral opening (Tan *et al*., 2006). Decreasing of *RhETR1* in petiole of high and low sensitive genotypes **(Fig.**

7, manuscript 1) is in contrast to the miniature roses cv. 'Bronze' and 'Vanilla'. In short flower life cultivar 'Bronze' and long-lasting cultivar 'Vanilla', the expression of *RhETR1* was clearly increased under ethylene treatment (Müller *et al*., 2000a and 2000b).

In the present study, the expression of *RhCTR1* and *RhCTR2* occurred constitutively in pedicels of all examined plants **(Fig. 6, manuscript 1)**. Similarly, the expression of two *CTR* genes did not show up-regulation in petal of cut roses after ethylene treatment (Tan *et al*., 2006). While, in petals of cv. 'Bronze', the expression of both *CTR* genes increased after ethylene treatment (Müller *et al*., 2002). In petiole samples, the expression pattern was different as *RhCTR1* was increased in genotype 76/67, while *RhCTR2* was decreased in genotype 76/74 **(Fig. 7, manuscript 1)**. The expression of *RhCTR2* in genotype 76/74 was suppressed by ethylene treatment. In contrast to this result *RhCTR2* increased in response to ethylene treatment in 'Bronze' and 'Vanilla' (Müller *et al*., 2002). These differences in gene expression may be due to tissue-specific expression pattern of studied genes. In this study, the gene expression was evaluated in petiole and pedicel, while in other studies northern blot was applied on RNA isolated from flowers parts specially petal.

Since these experimental data come from different tissues treated in different way by ethylene, it is difficult to explain them. However, our results **(Fig. 6 and 7, manuscript 1)** are not compatible with assumption that differences in display quality of rose cultivars are related to differences in receptor levels (Müller *et al*., 2000a and 2000b). Different reports on expression of ethylene receptor genes were published. In apple fruitlets, the expression pattern of *MdETR1*, *MdERS1*, and *MdCTR1* gene were related to tissue and/or population. Fruitlet populations were classified as their differences in abscission potential (Dal Cin *et al*., 2005). The accumulation of *ETR1* transcripts in peach did not change in various examined tissues and developmental stages by propylene, an ethylene analogue (Rasori *et al*., 2002). Also in non-rose family plants such as kiwifruit, the *ETR1* expression was negatively affected by endogenous and exogenous ethylene in ripening fruit (Yin *et al*., 2008). Ethylene receptor *ERS2* was down-regulated under ethylene treatment in young seedlings of rice (Yau *et al*., 2004), but it increased in sepals of *Delphinium* by ethylene (Tanase and Ichimura 2006).

Based on the published data on expression of ethylene receptor genes, it can be assumed that the plant response to ethylene cannot be explained only by expression pattern of ethylene receptor genes. Role of *EIN3*/*EIN3*-Like (*EIL*) family in the plant response to ethylene can support this claim. Loss of function mutation in EIN3 protein resulted in ethylene insensitivity in *Arabidopsis* (Solano *et al*., 1998) and tomato (Tieman *et al*., 2001). The transcription factor *RhEIN3* was constitutively expressed in response to ethylene in 'Vanilla' and 'Bronze' (Müller *et al*., 2003), whereas its expression in petals of cut rose cultivars, Samantha and Kardinal, was slightly enhanced by ethylene (Tan *et al*., 2006). Further studies are required to illuminate relationship between ethylene sensitivity and expression of genes acting as ethylene receptors and/or acting downstream in MAPK cascades. In evaluation of plant response to ethylene, developmental stage, tissue and genotypes characteristic are indispensable.

4.4 Isolation of ethylene induced cDNAs

In order to gain a broader understanding of the molecular basis of ethylene induced organs abscission process, DDRT-PCR method was applied on the leaf and bud of ethylene treated and non-treated rose plants. To our knowledge this is the first report about the application of DDRT-PCR method on the rose plants. Out of up-regulated 12 fragments in response to ethylene treatment, five transcripts showed sequence similarity to database **(Table 2, Ahmadi *et al.*, 2008)**.

In this study 88 ethylene up-regulated and 72 down-regulated bands were detected. The degrees of polymorphism for up-regulated and down-regulated fragments were 4.6% and 3.7% respectively. About 80% of up-regulated amplicons were false positive. Similar to our result, application of DDRT-PCR in other studies had resulted in a low number of isolated differentially expressed cDNA. For instance, nine transcripts were identified as being induced by salt stress in mangrove (Banzai *et al*., 2002) and similarly, nineteen ethylene-responsive cDNA clones isolated from tomato fruit (Zegzouti *et al*., 1999). The strengths and weaknesses of the DDRT-PCR were broadly discussed by Debouck (1995). Improving efficiency of this technique by avoiding of false positives and primer designing was the interesting topic for some biologist (Graf *et al*., 1997; Mohr *et al*., 1997; Sanabria and Dubery, 2004).

The other reason for detecting low number of differentially expressed transcripts in this research might be related to the time course of ethylene treatment for 72 h. Over time expression study on isolated laccase cDNA showed that its expression level was significantly detectable after 24 h **(Fig. 5, Ahmadi *et al.*, 2008)**. Long term ethylene treatment decreases the chance for detecting up-regulated cDNAs which expressed in the beginning of ethylene application. This idea could be supported when the mRNA accumulation levels of ethylene-responsive cDNA clones were clearly revealed after 15 min (Zegzouti *et al*., 1999) and 12 h (Harris *et al*., 1997) ethylene treatment in tomato. In ethylene-insensitive *etr1-3 Arabidopsis* mutant plants, 3 cDNAs were identified after 10 min following touch treatment. The touch treatment was performed by bending the plant leaves for 30 s by hand (Chotikacharoensuk *et al*., 2006). However, since the yellowing symptom appeared on bud pedicel after 6 days ethylene treatment, the ethylene treatment was applied for 3 days (72 h) in this study.

4.5 Induction, isolation and characterization of laccase gene

The sequence analysis of cDNA-5 showed highest similarity to laccase in *Zea mays* **(Table 2, Ahmadi *et al.*, 2008)**. The expression of cDNA-5 in petiole and pedicel of 'Lavender' was induced approximately 10 and 50 times respectively after ethylene treatment **(Fig. 1, Ahmadi *et al.*, 2008)**. Because of this distinctive expression, the complete sequence of this gene (termed *RhLAC*, accession No. EU603403) was isolated using 3' RACE-PCR method, and resulted in the first report on the up-regulation of laccase following ethylene treatment.

Multiple copies of the *RhLAC* gene existed in *Rosa* species **(Fig. 4, Ahmadi *et al.*, 2008)**. Same to our result, laccases in maize belong to a multigene family (Caparros-Ruiz *et al*., 2006). In higher plants, genome sequence studies have shown that multicopper oxidases related to laccase are widely distributed as multigene families (McCaig *et al*., 2005).

Laccases have been isolated from different organs like fungi and plants. Physical characterizations of the proteins show a great variety in molecular weight, carbohydrate content and quaternary structure (Germann and Lerch, 1986). Laccase is a multi-copper glycoprotein enzyme containing four copper atoms per protein molecule (Makino and Oura, 1971; Solomon *et al*., 1996).

4.6 Laccase expression in various organs and genotypes

The expression of *RhLAC* in leaf abscission zone that included small parts of the petiolar pulvinus and in bud abscission zone with a small segment of pedicel showed the highest level respectively. In contrast, the relative expression of *RhLAC* gene in ethylene-treated leaf blade and petiole was lower comparing to leaf and bud abscission zones **(Fig. 6, Ahmadi *et al.*, 2008).**

In this study, relative expression of *RhLAC* gene in ethylene to non-ethylene treated sample was analyzed using the $2^{-\Delta\Delta CT}$ method that represents the average relative amount of mRNA for each group by normalizing via the beta-actin expression (Livak and Schmittgen 2001). In our investigated samples such as leaf blade and petiole, low relative expression resulted from moderately high expression in non-ethylene-treated samples. The expression of *RhLAC* was studied in plants kept in glass chamber and greenhouse surrounded by ethylene-free air. *RhLAC* was expressed at a low rate in the plants located in glass chamber and no expression of *RhLAC* was detected in greenhouse plants (data not shown). It is supposed that endogenous ethylene was synthesized and induced *RhLAC* expression in non-ethylene-treated plants kept in glass chamber. Probably stress conditions such as high humidity in chambers induced endogenous ethylene production. However, since the present work took the first step in studying of laccase up-regulated by ethylene treatment, the expression of *RhLAC* gene must be specifically investigated and analyzed in different organs and tissues.

Transcript abundance of *RhLAC* in pedicels of high sensitive genotypes and ‘Lavender’ was significantly higher than in low sensitive genotypes and ‘Vanilla’. The lowest level of *RhLAC* expression was detected in pedicel of genotype 42/48 that showed lowest rate of bud abscission **(Fig. 8, manuscript 1)**. These results showed a relationship between relative expression of laccase and ethylene sensitivity in high and low sensitive genotypes. Further investigation is needed to verify this relationship.

4.7 Functional analysis of *RhLAC* by virus-induced gene silencing

Virus-induced gene silencing (VIGS) has been applied widely in plants for functional analysis of gene and has become accustomed as a powerful technique. VIGS is a method that takes advantage of the RNAi process. Acting as RNA-mediated antiviral defense mechanism, using virus vectors carrying inserts derived from host cause to initiate temporary knock-down of inserted genes in plants (Lu *et al.*, 2003).

To analyze *RhLAC* gene function, the VIGS tool was applied on *Nicotiana benthamiana*. The laccase gene was previously characterized from constructed cDNA from ethylene-treated and non-treated RNA isolated from *Rosa hybrida* **(Ahmadi *et al.*, 2008)**.

High efficiency was reported as the advantage of VIGS applications in *N. benthamiana,* regardless of used vector type (Xu *et al.*, 2007; Page *et al.*, 2004). The vectors derived from tobacco rattle virus (TRV) are widely used as VIGS vectors in various plants, especially for species of the *Solanaceae* family (Ratcliff *et al.*, 2001; Brigneti *et al.*, 2004; Dong *et al.*, 2007). Although some other vectors including tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) (Kumagai *et al.*, 1995), tomato golden mosaic virus (TGMV) (Kjemtrup *et al.*, 1998) and potato virus X (PVX) (Ruiz *et al.*, 1998) were used as VIGS vectors but these vectors brought some disadvantages. These vectors cause the common symptoms of leaf chlorosis and leaf distortion whereas the TRV vector induced a mild symptom that was not mistaken with VIGS phenotype (Ratcliff *et al.*, 2001). The other problem in using these vectors is related to the efficacy of vectors in tissue infection. TMV and PVX are unable to infect the apical meristem. Therefore they are not applicable in the studies of genes involved in development of plant tissues and organs (Chen *et al.*, 2004). In contrast to PVX, the TRV infected systematically wide tissue area and passed through the growing points resulting in silencing expression of genes in the meristem (Ratcliff *et al.*, 2001).

As non-sense fragment (control), *N. benthamiana* plants were inoculated with TRV2 carrying partial sequence of glucuronidase gene. This exogenous gene does not exist in plant genome and logically does not cause any changes in plant biochemical pathways.

The phytoene desaturase (*PDS*) reporter gene was used in this experiment. Six to seven days after infiltration, the photobleaching symptoms resulting from *PDS* silencing

developed on freshly emerged leaves. Infiltration of photosynthetic tissues with TRV carrying a *PDS* segment decreased the abundance of *PDS* transcript and subsequently resulting in leaf photobleaching (Chen *et al.*, 2004). Phytoene desaturase plays role in carotene formation pathway. Carotenoids are essential elements for plant survival by protection of chlorophylls against photooxidation (Wagner *et al.*, 2002). We supposed that in this study the TRV vectors spread systematically through the plant in less than 6 days and inoculate apical or secondary meristems. This supposition would be supported by some findings. It was showed that the distribution of TRV vectors in the root of inoculated *N. benthamiana* was detected after 4-6 days of postinoculation by green fluorescent protein used as genetic marker (Valentine *et al*., 2004). Similarly, the symptom of mild mosaic was developed on the leaves infected with pBINTRA6, an RNA1 Ti plasmid clone of TRV, 5 days after infiltration in *N. benthamiana* plants (Ratcliff *et al.*, 2001; Lu *et al.*, 2003). Regarding these data, the plants were treated with ethylene 5 days after inoculation.

Exogenous application of ethylene significantly decreased plant height **(Fig. 2, manuscript 2)**. This agrees with Anten *et al*. (2006) who suggested ethylene involvement in growth inhibition of tobacco (*Nicotianum tabacum*) shoots. Ethylene treatment decreased the total number of leaves **(Fig. 3, manuscript 2)**, and the total green leaves area per plant **(Fig. 4, manuscript 2)**. Effect of ethylene on cell division and cell elongation was studied on transgenic tobacco plants. It was showed that ethylene is a negative regulator of cell elongation in 35S:NtENOD40 tobacco cells. Overexpression of *NtENOD40* mediated by ethylene accumulation reduced cell elongation (Ruttink *et al*., 2006). Early nodulin (*ENOD*) genes are specifically induced by nodulation factor- secreting rhizobia during early stages of nodule development in plants.

Ethylene treated *N. benthamiana* plant did not show leaf abscission. Leaves turned yellow and died while attached to the main body of the plant. Similarly to *N. benthamiana*, 8-week-old seedlings of *Nicotiana tabacum* did not exhibit abscission (Aharoni, 1978). The non-abscission type of leaves in *N. benthamiana* seedlings is a main problem of using this plant in VIGS study of genes related to the leaf abscission process.

Measuring plants height and green leaves area showed that the major effect of laccase gene silencing appeared as plant dwarfing, compare with control plants **(Fig. 2 and 4, manuscript 2)**.

It is difficult to interpret these results without confirmation of laccase homologue gene(s) silencing in *N. benthamiana*. Characterization of 17 laccase homologue genes with different functions in *Arabidopsis thaliana* (Cai *et al*., 2006) reveals the necessity of performing northern blot and quantitative PCR to shed light on the obscure molecular part of laccase-silencing process in this research. Molecular analysis becomes more important when we are dealing with multigene families like laccase **(Ahmadi *et al*., 2008).**

Our results **(Fig. 2 and 4, manuscript 2)** show that the silencing laccase homologue retarded growth and development of plant. So far, many functions have been attributed to laccase including lignin biosynthesis (Bao *et al*., 1993; Sterjiades *et al*., 1992; Ranocha *et al*., 1999), wound-healing as part of an herbivore or pathogen defense response, pigment formation and detoxification (Solomon *et al*., 1996; Mayer and Staples 2002; McCaig *et al*., 2005; Pourcel *et al*., 2005). The expression of laccase in differentiating xylem tissue could be assigned to lignin synthesis in developing xylem (Sato *et al*., 2001). In cocoa plants, differentially expression of laccase-like genes is related to inducing plant resistance to witches´ broom disease caused by *Basidiomycete* fungus *Crinipellis perniciosa* (Leal *et al*., 2007).

Although various types of laccases with noted functions were identified, however suppression of laccase in herbaceous tissue (**manuscript 2)** and its expression under ethylene **(Ahmadi *et al*., 2008)** implies some other physiological functions for laccase.

Based on our data, we suppose that laccase affects somehow plant cell elongation and/or cell division. Findings on several laccases genes characterized in some plants strengthen this presumption. De Marco and Roubelakis-Angelakis (1997) suggested that laccase is involved in cell wall formation without playing role in lignifications. They detected the increasing of laccase activity in tobacco regenerating protoplast. Four kinds of laccase mRNAs isolated from maize were accumulated in root elongation zone; although the expression of cDNA homologue to our investigated laccase was lower than others (Caparros-Ruiz *et al*., 2006). In ryegrass (*Lolium perenne*), expression of laccase

gene (*LpLAC*2-1) was at high level in meristimatic tissues and stem and it was at low level in roots. However, expression of laccase was not detected in flower and leaf samples (Gavnholta *et al*., 2002).

The partial sequences of laccase used in this study shows 61% homology to laccase 15 in *Arabidopsis thaliana*. Laccase 15 plays role in oxidative polymerization of flavonoids in the seed coat and root elongation (Pourcel *et al*., 2005; Cai *et al*., 2006; Liang *et al*., 2006a). Thus, extending the experimental course to flowering stage and seed production would be necessary to see any laccase silencing effect on seed color. The effect of laccase silencing has to be evaluated in root elongation too.

Our report pointed out the induction of laccase gene expression under ethylene treatment **(Ahmadi *et al*., 2008)**. Also transcript level of laccase mRNA increased after application of ABA in tomato *(Lycopersicon esculentum*) roots (Wei *et al*., 2000). Expression of laccase by ethylene and ABA may confirm the relationship between both plant hormones. The interaction between ethylene and ABA is relatively complex, as reciprocal effects have been demonstrated. A close relationship between ABA and ethylene was especially observed in abscission process. ABA treatment induced ethylene production and subsequently abscission in different organs in rose (Mayak and Halevy 1972, Müller *et al*., 2001b) as well as in other plants (Suttle and Hultstrand 1993; Chen *et al*., 2002). In *Phaseolus vulgaris*, ABA application induced ethylene production and promoted explants abscission (Jackson and Osborne, 1972). In contrast, auxin-induced ethylene up-regulates ABA biosynthesis at the transcription level in cleavers (*Galium aparine*) (Kraft *et al*., 2007). Similarly, in avocado (*Persea americana* Mill. cv Lula) as a climacteric fruit, increasing in ethylene biosynthesis is accompanied with rising in ABA (Chernys and Zeevaart, 2000). ABA is synthesized via cleavage of xanthophylls to xanthoxin, catalysed by 9-cis-epoxycarotenoid dioxygenase (NCED). Then xanthoxin is converted into ABA through ABA-aldehyde (Chernys and Zeevaart, 2000; Kraft *et al*., 2007). Some indirect evidences showed that the cleavage reaction is the bottle neck in ABA synthesis. Two genes encoding NCED were strongly expressed in ripening avocado fruit (Chernys and Zeevaart, 2000).

Inhibitory effect of ethylene was more severe on *LAC* silenced gene plants than on control plants **(Fig. 2 and 4, manuscript 2)**. Increasing laccase expression under ethylene treatment or other biotic or abiotic stress conditions (Liang *et al*., 2006b; Wei

et al., 2000) may be related to plant defense mechanism in reaction to stress conditions. It can be presumed that the accumulation of laccase prepares the plant to encounter stressful situations. Increased relative expression of *RhLAC* in high sensitive genotypes than in low sensitive genotypes **(Fig. 8, manuscript 1)** suggests that high sensitive genotypes recognized detrimental conditions by greater expression of *RhLAC* than low sensitive genotypes. In other view, laccase silenced plant is disarmed to react well to ethylene treatment. Therefore, ethylene is more effective on the laccase silenced plants than on the control plants **(Fig. 2 and 4, manuscript 2)**. Certainly, these data are not sufficient to explain the laccase expression and function under ethylene treatment.

In conclusion, ethylene-induced *RhLAC* gene was isolated from the miniature rose by DDRT-PCR. This gene that belongs to multigene family encodes a putative protein containing 573 amino acids. The main effects of laccase silencing were revealed in retardation of growth and development of *N. benthamiana*. Under ethylene treatment, the plant size and total green leaves area in laccase silenced plants were affected more drastically than control plants. We suppose that laccae participates somehow in cell division and (or) cell enlargement. Considering that organ abscission induced by various abiotic and biotic stress conditions (Addicott, 1982), ethylene-induced laccase could be involved in abscission process. Undoubtedly, these premature ideas need to be evaluated carefully, especially because we deal with a big multiple gene family playing various physiological functions.

4.8 Outlook

In these studies, the reaction of F1 breeding lines to exogenous ethylene was screened and 2 genotypes with high sensitivity and 3 genotypes with low sensitivity were selected. Using DDRT-PCR, 17 cDNA fragments were cloned, out of them 7 cDNAs did not show homology to database. Silencing laccase homologue gene stunted plant growth by reducing plant size and total green leaves area. It has to be improved the silencing of laccase gene. Also further experiments should be carried out to answer some questions lying behind presented in this thesis investigation:

- Molecular analysis of plants of VIGS experiment for verifying silencing of laccase homologue genes.

- Identification and expression analysis of 7-unknown cDNA fragments isolated by DDRT-PCR.
- Elucidation of the linkage between *RhLAC* expression and genes involved in ethylene synthesis, receptors and signal transduction pathway.
- Detail study on relationship between laccase expression and abscission behavior in other rose cultivars, progenies and genotypes.
- Evaluation of ethylene and ABA effects on laccase expression separately or simultaneously.

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Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation

Characterization of Ethylene-Induced Abscission in Miniature Rose (*Rosa hybrida* L.)

selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden. Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 28.10. 2008

Noorollah Ahmadi

Lebenslauf

Name:	Noorollah Ahmadi
Geboren:	11.09.1969 in Eghlid, Iran
Staatsangehörigkeit:	Iranisch
Familienstand:	verheiratet/ 2 Kinder
Adresse:	Bischofsholer Damm 85 (Woh. 444), 30173 Hannover

Studium

seit 02/2004	PhD. Student an der Gottfried Wilhelm Leibniz Universität Hannover
1997-2000	MSc. im Gartenbau, Tarbiat Modares Universität,Teheran, Iran
1995-1997	BSc. in der Agrartechnik, Universität Teheran, Teheran, Iran
1988-1990	Studium der Agrartechnik, Universität Sistan & Balouchestan, Zabol, Iran
1990	Abitur in experimentellen Wissenschaften (High school diploma), Shiraz, Iran

Ehrungen und Auszeichnungen:

1997	Landes beste Eingangsprüfung zur Aufnahme zum Master Studium an den Üniversitäten im Iran.
1997-1999	Stipendium der Landwirtschaftlichen Forschungs- und Ausbildungs-Organisation des Iran zur Finanzierung des Master Studiums an der Tarbiat Modares Universität.
1999	Mitglied des Wissenschaftlichen Organisationskommitees der Konferenz „3th National Conference on Olive '*Olea europaea*'", im Oktober 1999 in Karaj, Iran
2004- 2008	Stipendium des Ministeriums für Wissenschaften, Forschung und Technologie des Iran zur Finanzierung des Promotionsstudiums im Ausland.
Berufserfahrung: (2000-2003)	Wissenschaftler am Fars Forschungszentrum für Landwirtschaft und natürliche Ressourcen in Jahrom, Iran
Auslandpraktika:	4-wöchiges Praktikum an der Leibniz Universität Hannover, Institut für Pflanzengenetik, Abteilung Pflanzenbiotechnologie in der Arbeitsgruppe von Herrn Prof. Jacobsen, 21.02.-18.03.2005.
Mitgliedschaften:	
	Mitglied der Internationalen Gesellschaft für Gartenbau "International Society for Horticultural Science"

Publications

Ahmadi, N., Arzani, K., Moeini, A. (2001). Study of pollen storage, germination and pollen tube growth in some citrus cultivars. Seed and Plant Journal (SPII), Vol 17, No (1):216- 229. (In Persian with English Abstract)

Ahmadi, N., Mibus H., Serek M. (2008). Isolation of an ethylene induced putative nucleotide laccase in miniature roses (Rosa hybrida L.). J. Plant Growth Regul. 27: 320-330.

Ahmadi, N., Mibus, H. and Serek, M. Characterization of ethylene-induced organ abscission in F1 breeding lines of miniature roses (*Rosa hybrida* L.). Postharvest Biology and Technology (in press)

Ahmadi, N., Mibus, H. and Serek, M. Identification of ethylene induced genes in abscission zone of *Rosa hybrida* L. by use of differential display. 9th International Symposium on Postharvest Quality of Ornamental Plants 10-14.06.2008, Odense, Denmark. Acta Horticulture (in press)