J Plant Growth Regul (2008) 27:320–330
DOI 10.1007/s00344-008-9059-2

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

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Received: 29 October 2007 / Accepted: 13 June 2008 / Published online: 20 August 2008

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 (*CTR*s) 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).

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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

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.

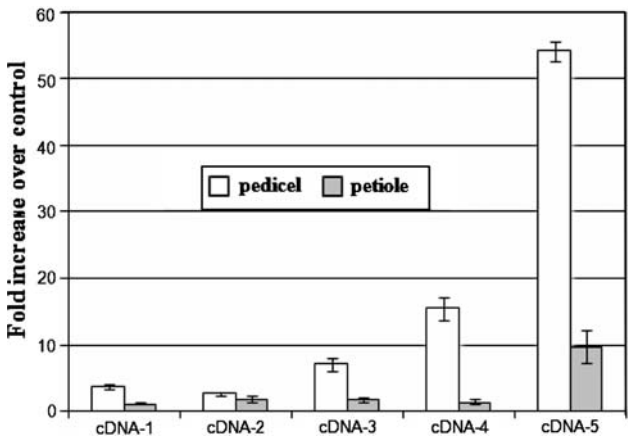

Fig. 1 Relative expression of the isolated cDNA genes induced in pedicel and petiole of rose after 72 h of ethylene treatment. The fold-change expressions of the isolated genes were calculated relative to the untreated sample as control after normalization to the β-actin gene. Expression level in untreated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

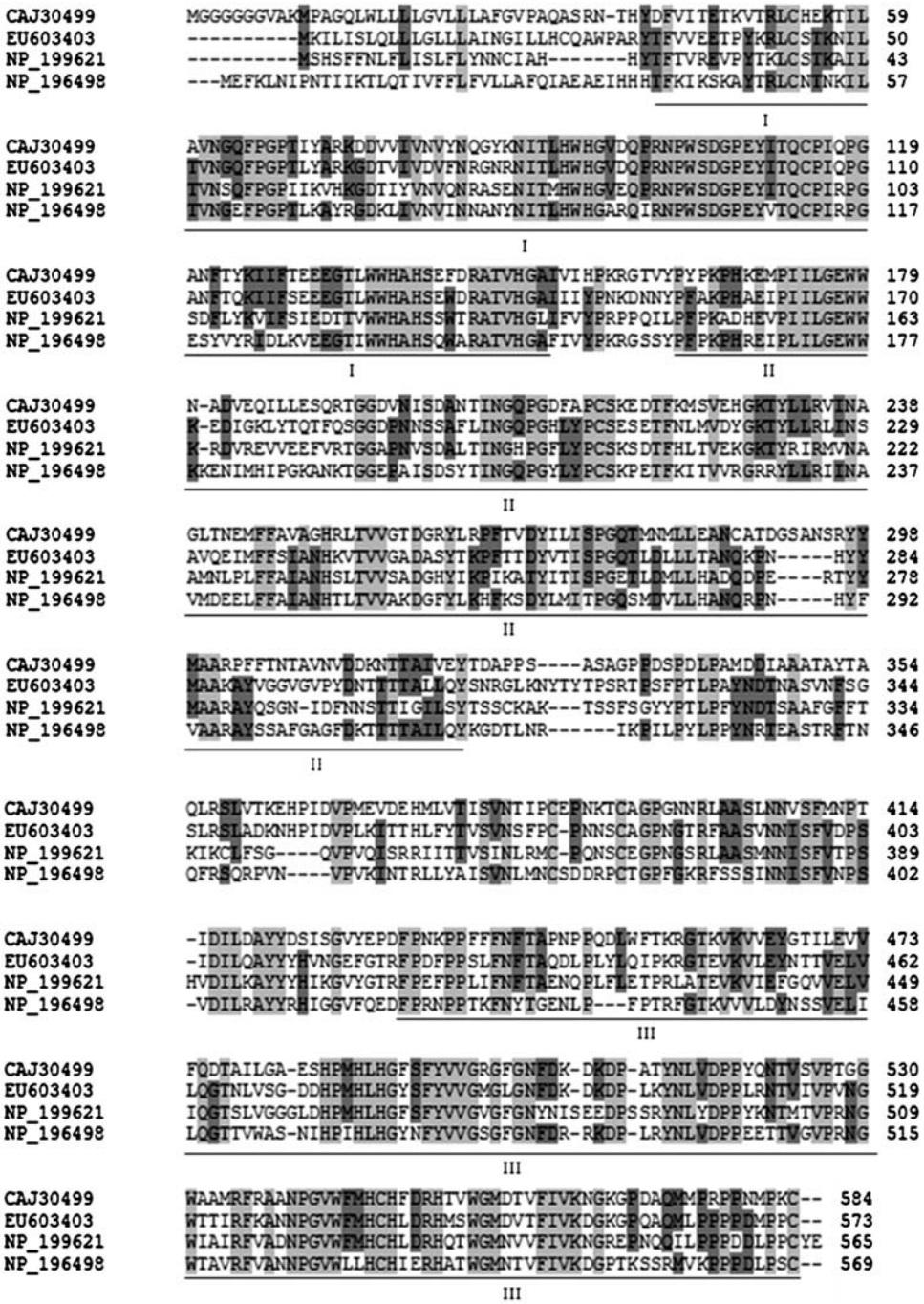

Fig. 2 Comparison of the deduced amino acid sequence laccase from *Rosa hybrida* (accession No. EU603403) with putative laccase from *Zea mays* (accession No. CAJ30499), copper ion binding from *Arabidopsis thaliana* (accession No. NP_196498), and TT10 (TRANSPARENT TESTA 10) laccase from *Arabidopsis 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

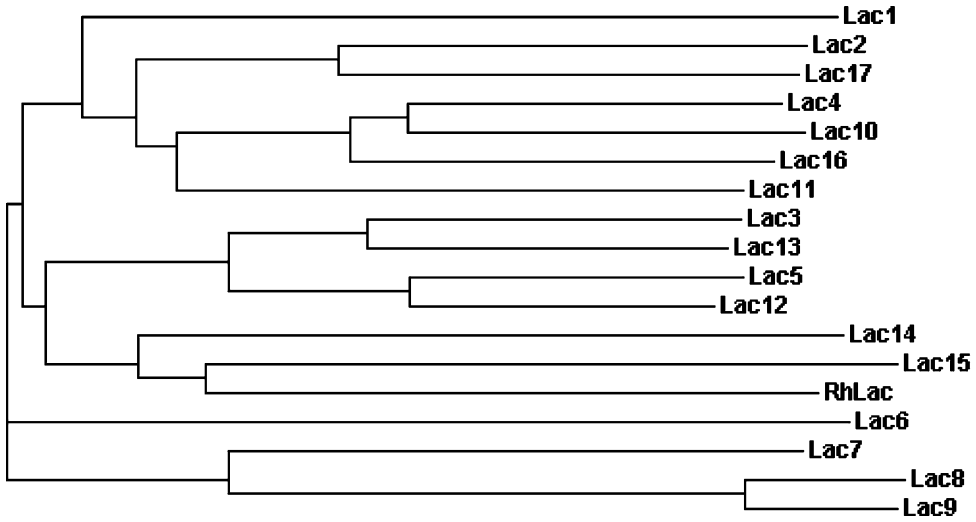

Fig. 3 Phylogenetic analysis of *RhLAC* with 17 laccases isolated from *Arabidopsis thaliana.* Sequence alignment analysis was performed using a CLUSTAL W method. The following are the corresponding accession numbers for each protein: AtLAC1 (NP_173252), AtLAC2 (NP_180477), AtLAC3 (NP_180580), AtLAC4 (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

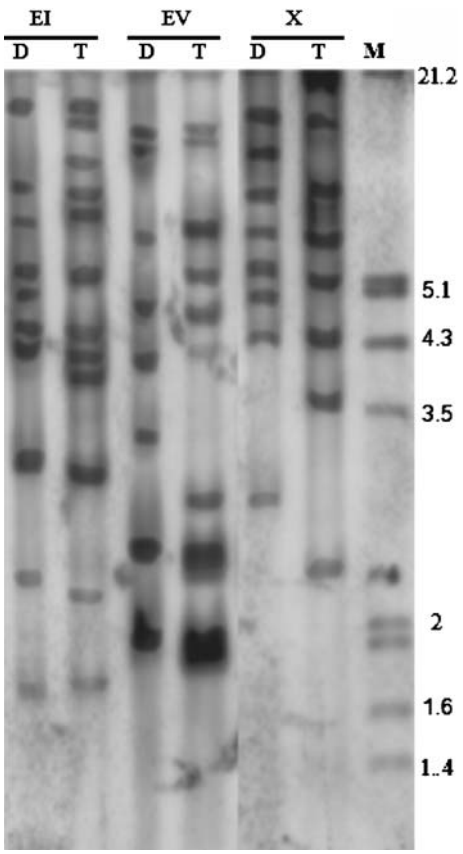

Fig. 4 Southern blot hybridization analysis of the *RhLAC* gene. The genomic DNA of diploid rose genotype 94/1 (D) and tetraploid *Rosa hybrid* L. cv. Lavender (T) were digested with *Eco*RI (EI), *Eco*RV (EV), and *Xba*I (X). M = DIG-labeled DNA Molecular Weight Marker III

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

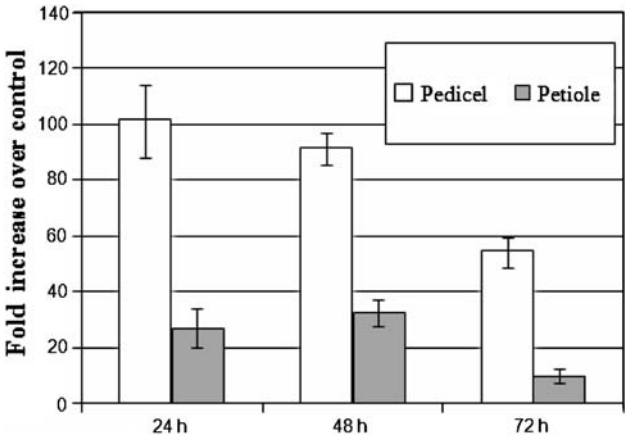

Fig. 5 Relative expression of the *RhLAC* gene induced in pedicel and petiole of rose after 24, 48, and 72 h of ethylene treatment. The fold-change expression of the isolated gene was calculated relative to the untreated sample as control after normalization to the β-actin gene. Expression level in untreated 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 (homeo-box-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

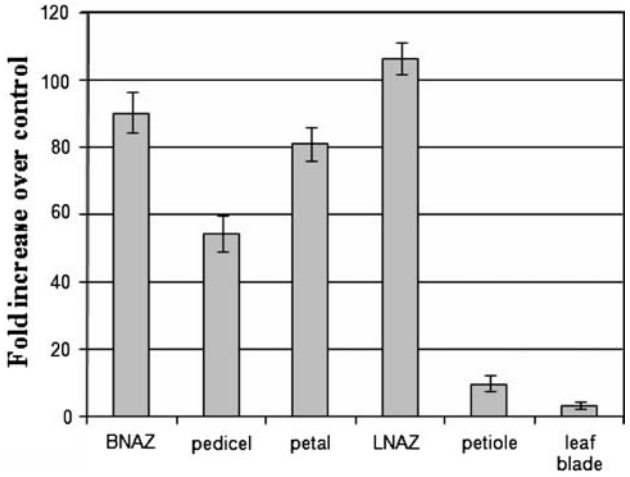

Fig. 6 Relative expression of the *RhLAC* gene expressed under 72 h of ethylene treatment in the bud abscission zone together with 2 mm of the proximal region of floral bud pedicel (BANZ), pedicel, petal, leaf abscission zone together with 2 mm of petiolar pulvinus (LANZ), petiole, and leaf blade. The 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.

Acknowledgments This study was supported by a PhD grant (to NA) from the Ministry of Science, Research and Technology of Iran. The authors would like to thank Prof. Bjarne M. Stummann for critical reading of the manuscript and valuable comments, and Prof. Errol Hewett (Massey University, Palmerston North, New Zealand) for linguistic editing of the manuscript.

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