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Characterization of expression and enzyme activity of lipoxygenases during fruit softening and superficial scald development in ‘Wujiuxiang’ pear

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Summary

Lipoxygenases (LOXs; EC 1.13.11.12) have been implicated in fruit ripening and senescence; however, little is known regarding the specific LOX genes involved in fruit softening and scald development in pear. In this study, two 9-lipoxygenase genes, *PcLOX1* and *PcLOX7*, were characterized *in silico* in the *Pyrus communis* draft genome. The expression pattern of *PcLOX1* and *PcLOX7*, LOX activity, respiration rate and ethylene production were analyzed during fruit softening and superficial scald development in ‘Wujiuxiang’ pear with or without 1-MCP treatment. The results demonstrated that *PcLOX1* and *PcLOX7* belong to the type-1 LOX subfamily, and their expression levels, enzyme activity, respiration rate and ethylene production were increased during fruit softening and scald development. In contrast, 1-MCP treatment could effectively suppress respiration rate and ethylene production and inhibit expression of *PcLOX1* and *PcLOX7* and LOX enzyme activity, resulting in delayed fruit softening and scald development. These findings indicate that *PcLOX1* and *PcLOX7* are involved in fruit ripening and scald development in ‘Wujiuxiang’ pear.

Abbreviations: LOX, lipoxygenase; PUFA, polyunsaturated fatty acid; 1-MCP, 1-methylcyclopropene

Introduction

Lipoxygenases (LOXs; EC 1.13.11.12), a group of non-heme iron-containing dioxygenases, catalyze the addition of oxygen to either end of a (Z, Z)-1,4-pentadiene moiety of polyunsaturated fatty acids (PUFAs) to produce an unsaturated fatty acid hydroperoxide (BRASH, 1999). Linolenic and linoleic acids are the most common substrates for plant LOXs, which can be divided into 9-LOXs and 13-LOXs according to the region-specificity of the oxygen added, leading to 9- or 13-hydroperoxy fatty acids (FEUSSNER and WASTERNAK, 2002). Based on protein structure, plant LOXs can also be classified into two subfamilies, type-1 and type-2. Type-1 enzymes harbor no transit peptide, and type-2 enzymes carry a plastid transit peptide sequence (LIAVONCHANKA and FEUSSNER, 2006).

LOXs have been shown to have multiple functions in plants, such as involvement in biotic or abiotic stress (HU et al., 2015; HWANG and HWANG, 2010; LIM et al., 2015; LÓPEZ et al., 2011), production of volatiles and flavor compounds (ALTISENT et al., 2009; CHEN et al., 2004; LI et al., 2016), fruit ripening (GRIFFITHS et al., 1999; HAN et al., 2011; LI and WANG, 2009; LV et al., 2014; MENG et al., 2016; KAUSCH and HANDA, 1997), and plant senescence (GOMEZ-LOBATO et al., 2012; LIU et al., 2013; SELTMANN et al., 2010; SPRINGER et al., 2016). It has been suggested that fruit ripening and plant senescence may be attributable to membrane deterioration and peroxidation of PUFAs, resulting in loss of cellular compartments and cell breakdown (BAYSAL and DEMIRDÖVEN, 2007).

Horticulture plants are raw material and used by people for food, either as edible products, or for culinary ingredients, for medicinal use or ornamental and aesthetic purposes. Fruits and vegetables are an important component of traditional food, but are also central to healthy diets of modern urban population (FENG et al., 2014; HRICOVÁ et al., 2016; MLČEK et al., 2015). The ‘Wujiuxiang’ (*Pyrus communis* L.) pear is favored for its excellent flavor quality, and it therefore exhibits a high market value. However, as a climacteric fruit, this pear may quickly soften after several days at ambient temperature, which makes it difficult to transport and store, resulting in reduced fruit quality and economic loss (GUAN et al., 2014). In addition, it is highly susceptible to superficial scald, a physiological disorder that manifests as many irregular brown or black patches on the fruit skin during cold storage and subsequent shelf life (DONG et al., 2012; GAO et al., 2015). It has been shown that ethylene has a pivotal role during these senescence processes, while 1-methylcyclopropene (1-MCP) can effectively delay fruit ripening and scald development in apples and pears (GAO et al., 2015; GUAN et al., 2014). However, the mechanisms underlying these senescence processes are not fully understood. Several studies have demonstrated that LOX enzyme activity may be related to fruit ripening and scald development in pears (FEYS et al., 1980; GAO et al., 2015; LI and WANG, 2009; LIU et al., 2013). However, due to the large size of the LOX gene family (ANDREOU and FEUSSNER, 2009), in which 23 LOX genes were found in the pear genome (LI et al., 2014), little is known regarding the role of specific LOX family members during fruit ripening and scald development in pear fruit.

It has been shown that *LOX1* and *LOX7* were strongly expressed in apples during storage (VOGT et al., 2013), implying involvement in fruit ripening and senescence. In this study, *PcLOX1* and *PcLOX7* were characterized *in silico*. In addition, *PcLOX1* and *PcLOX7* expression and LOX enzyme activity were analyzed during fruit ripening and scald development in ‘Wujiuxiang’ pears with or without 1-MCP treatment. The results showed that both *PcLOX1* and *PcLOX7* expression and LOX enzyme activity were related to fruit ripening and scald development, indicating the involvement of LOXs during senescence in ‘Wujiuxiang’ pear.

Materials and methods

Materials and treatments

‘Wujiuxiang’ pear fruit were harvested in Jinzhou County (Hebei, China) at commercial maturity stage (September 4, 2013). The harvested fruit were transported to the laboratory within 2 h. Fruit that exhibited uniformity of weight, shape and color without any visual defects were selected. For the fruit ripening experiment at ambient temperature (EXP. 1), the fruit was randomly divided into two lots. One lot was exposed to 1.0 µL/L 1-MCP (Rohm and Haas China Inc., Beijing) for 24 h at 25 ± 2 °C. Another lot was sealed with air and used as the control. After treatment, each group was stored at 25 °C for the fruit ripening experiment. For the cold storage and shelf life experiment (EXP. 2), 1-MCP treatment was conducted as

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in EXP. 1. After treatment, the fruit was stored at 0 °C for 120 days. After 120 days of cold storage, the fruit was transferred to 25 °C for 7 days of shelf life.

Respiration rate

Respiration rate was measured using an infrared carbon dioxide analyzer (Kexi Instruments, Jiangsu, China; Model HWF-1A) at 0 °C after fruit were sealed in glass desiccators (9.35 L) for 30 min. Next, a 1-mL sample of gas was withdrawn with a syringe to analyze the respiration rate. The result was expressed as mg CO₂ kg⁻¹ FW h⁻¹. Three replicates were measured for each treatment, with 10 fruit per replicate.

Ethylene production rate

The pear fruit were randomly selected and sealed in glass desiccators (9.35 L) for 4 h at 0 °C. A 1-mL sample of the headspace gas was withdrawn with a gas-tight syringe from each desiccator through a septum stopper and injected into a gas chromatograph (Kechuang Instruments, Shanghai, China; Model GC-9800) that was equipped with a GDX-502 column and a flame ionization detector (FID). The column temperature was 78 °C, and the injection temperature was 120 °C. The carrier gas was N₂ with a rate of 40 mL min⁻¹, and the rate of ethylene production was expressed as μL kg⁻¹ FW h⁻¹. Three replicates were measured for each treatment, with 10 fruit per replicate.

Scald incidence measurement

Superficial scald was assessed according to WHITAKER et al. (2009). Scald incidence was defined as the percentage of fruit exhibiting scald symptoms on the fruit surface. Three replicates were measured for each treatment with 30 fruit per replicate.

Firmness

Flesh firmness was measured at two points on the equatorial surface of each fruit after peel removal using a fruit hardness tester (TuoPu Instruments, Zhejiang, China; Model GY-J) mounted on a standard drill press and fitted with an 8-mm probe.

Bioinformatics analysis of *PcLOX1* and *PcLOX7*

The sequences of *PcLOX1* (PCP003542) and *PcLOX7* (PCP003539) were obtained from the *Pyrus communis* draft genome v1.0 hybrid transcripts database in Genome Database for Rosaceae (<https://www.rosaceae.org>). Deduced protein sequences were translated from full length coding sequences using EditSeq in the DNASTAR software package. Multiple sequence alignment of *PcLOX1* and *PcLOX7* with LOX proteins from other species was carried out using DNAMAN 8.0 software. Conserved domains were analyzed using the PROSITE program (<http://prosite.expasy.org/>). To determine the relationship between *PcLOX1*, *PcLOX7* and other LOX proteins, phylogenetic analysis was conducted for eighteen LOX proteins of different species using MEGA 6.05 by the neighbor-joining method with 1000 bootstrap replicates (TAMURA et al., 2013).

RNA isolation and quantitative RT-PCR

Total RNA was extracted using 'easyspin plant RNA extraction' reagent (Biomed, China) according to the manufacturer's instructions; all RNA extracts were treated with DNaseI (TaKaRa Biomedicals, Japan) and later purified following the manufacturer's instructions. First-strand cDNAs were synthesized from DNase-treated RNA (0.5 μg) using TaKaRa RNA PCR Kit (AMV) Version 3.0 (TaKaRa Biomedicals, Japan). Quantitative PCR was performed using SYBR

Premix Ex TaqTM (perfect Real Time) Kit (TaKaRa Biomedicals, Japan) on a 7500 Real-Time PCR system (Applied Biosystems, USA). PCR primers were designed using OMIGA 2.0 based on the sequences of *PcLOX1* and *PcLOX7*. The *PcLOX1* transcript was amplified using the forward primer 5'-GCTGGAATCCTTGTTTC-GACC-3' and the reverse primer 5'-CAATAAACCTTCAGGCA-ACGG-3', and the *PcLOX7* transcript was amplified using the forward primer 5'-CTTCCAAGTTTTTCGATGGAATG-3' and the reverse primer 5'-TGACACGCTTGAATCTTCAACC-3'. The *actin* gene transcript served as an internal control and was amplified using the forward primer 5'-GGTAACATTGTGCTCAGTGGTGG-3' and the reverse primer 5'-AACGACCTTAATCTTCATGCTGC-3'. The qRT-PCR reaction was carried out in a final volume of 20 μL containing 10 μL SYBR Green PCR Premix Ex TaqTM, 1 forward and 1 reverse primer, and 10 ng cDNA. PCR cycles were performed as follows: 10 s at 95 °C, 40 cycles of 95 °C for 5 s and 60 °C for 34 s. To confirm the specificity of amplification, the melting temperatures of amplification products were determined based on dissociation curve analysis. All qRT-PCR reactions were normalized using a Ct value corresponding to the *Actin* gene, the relative expression levels of target genes were calculated using the formula 2^{-ΔΔCT}, and the experiment was performed with three replicates.

Extraction and activity measurement of LOXs

The extraction and measurement of LOXs (EC 1.13.11.12) were performed according to AXELROD et al. (1981) with some modifications. Two grams of frozen peel were ground in liquid N₂ with a mortar and pestle. The completely ground powder was suspended in 5 mL of 100 mM phosphate buffer (pH 7.5), 2 mM DTT, 1 mM EDTA, 0.1% (v/v) Triton X-100, and 1% (w/v) polyvinylpyrrolidone (PVPP). The extract was centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was transferred to fresh tubes as the crude enzyme extract for the measurement of LOX activity.

LOX activity was assayed using sodium linoleate as a substrate. The substrate was prepared as follows: 28 μL of sodium linoleate and 36 μL of Tween-20 were mixed in 8 mL of oxygen-free water, and 2 M NaOH was added to clear the solution. Finally, the volume of the solution was brought to 10 mL with distilled water. The assay mixture (3 mL) contained 2.925 mL of 200 mM phosphate buffer (pH 7.0), 25 μL of the substrate solution and 50 μL of the crude enzyme extract. The reaction was initiated by the addition of crude enzyme extract. The absorbance of the mixture was recorded at 234 nm every 1 min for 8 minutes at 25 °C. LOX activity is expressed as ΔOD₂₃₄ min⁻¹ g⁻¹ FW.

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) according to treatment. All values are expressed as the mean ± SE of three replicates. Differences were considered significant at *P* < 0.05. All analyses were performed with SPSS 12.0 software (SPSS Inc. Chicago, IL, USA).

Results

Characterization of *PcLOX1* and *PcLOX7*

The CDS of *PcLOX1* and *PcLOX7* are 2607 and 2589 bp, respectively, encoding proteins of 868 and 862 amino acids, respectively. Further analysis of the deduced amino acid sequences using the PROSITE program revealed that *PcLOX1* and *PcLOX7* each contain a conserved PLAT domain and a lipoygenase domain with putative iron binding sites (His523, His528, His714, Asn718, and Ile862 for *PcLOX1*; His529, His534, His720, Asn724, and Ile868 for *PcLOX7*). *PcLOX1* and *PcLOX7* share 70.5% identity with each other, while *PcLOX1* and *PcLOX7* share 72.6% and 63.5% identity with *Arabi-*

| | | |
|---------|---|-----|
| PcLOX1 |MLQNIYEKLTG...HQQNGNHG.KTSEVIVLWKKNVLDNF | 38 |
| PcLOX7 |MLHSKYPTSSQDITVDTQPKNEGKRIKGRVLMKKVLDVNDL | 43 |
| AtLOX1 |MRGELRDLITG...GGNETTKRVKGRVLMKKVLDVNDF | 48 |
| MdLOX7a |MLHSKYPTSSQDITVDTQPKNEGKRIKGRVLMKKVLDVNDL | 43 |
| VvLOX |MHISVGAITG...ENDK...KIKIGVLMKKVLDVNDF | 35 |
| PdLOX |MLHNLFDKITG...QEQNGKGRKIKGTVLMKKVLDVNDF | 39 |
| AtLOX6 | ..MFVASPVKTFNFGVSLVKSPAFSALSCRQHRVPSISRQVRAVISREKAVDQEDGKSTKPLINSSQFPWRQSKYTGSKTWTYVVKRKKIKKELKTR | 99 |
| MdLOX8b | MYAVKQSTASLKSAAVGRVTPGPNYTRVRAKHPGSRVNGGQSVRAAISGGDKVTYTAATPLQSKGVDKLSSSGGGELQKAVVTRKMKKELKTR | 100 |
| | | |
| PcLOX1 | NASLDRVHLLVQGVSLCLVSAVHADSENGLQALGKPAYLEDVITTTPLTAGESAKVTFJWEVEVCPGAFILKNNHHSFVLRKTLVLEDFVGG | 138 |
| PcLOX7 | KASLDRVHLLVQGVSLCLVSSVNGD.PENELGKVGKPAYLENVITTTPLTAGEAADVTFJWDKEIGVPGAFVVRNHHHSFVLRKTLVLEDFVGG | 142 |
| AtLOX1 | NASLDRVHLLVQGVSLCLVSSVNGD.SENGLQALGKAHLEDVITTTPLTAGESAKVTFJWEVEVCPGAFILKNNHHSFVLRKTLVLEDFVGG | 137 |
| MdLOX7a | KASLDRVHLLVQGVSLCLVSSVNGD.PENELGKVGKPAYLENVITTTPLTAGEAADVTFJWEKEIGVPGAFVVRNHHHSFVLRKTLVLEDFVGG | 142 |
| VvLOX | NASVDRVHLLVQGVSLCLVSAVHADG.PANGLQALGKPAYLEDVITTTPLTAGESAKVTFJWEVEEIGVPGAFILKNNHHSFVLRKTLVLEDFVGG | 134 |
| PdLOX | NASVDRVHLLVQGVSLCLVSAHDGL.SENGLQALGKPAYLEDVITTTPLTAGESAKVTFJWEVEEIGVPGAFILKNNHHSFVLRKTLVLEDFVGG | 138 |
| AtLOX6 | FEHQDLFMKATLQGMILCLVSEEDP...ETGGRKSLSPAMGPKAKDDP...RYLVITADGTVPINFGCPGAILVTLNLSLSTGISELITLSDS...TD | 192 |
| MdLOX8b | IEDQVFFNIGLQGGILCLVSEQVDP...VTNSGKIVQSVVRVWPKPVPSEYHIVIEAADGTVPDFGCPGAILVTLNLSLSTGISELITLSDS...HGFDFG | 196 |
| | | |
| PcLOX1 | RHVFVONWYPAKIKTKRHFVFNKTYLSEFPLPRVYRFEELHHRGDL...KGLQEWFRVYDYVNDLGNPKGAKYVRPVGSSVYPFRGR | 236 |
| PcLOX7 | RHVFVONWYPAKIKTKRHFVFNKTYLSEFPLPRVYRFEELHHRGDL...KGLQEWFRVYDYVNDLGNPKGTEYARPVGSSVYPFRGR | 242 |
| AtLOX1 | RHVFVONWYPAKIKTKRHFVFNKTYLSEFPLPRVYRFEELHHRGDL...EGLEKEDRVYDYVNDLGNPKP...RVPVGTQVYPFRGR | 232 |
| MdLOX7a | RHVFVONWYPAKIKTKRHFVFNKTYLSEFPLPRVYRFEELHHRGDL...KGLQEWFRVYDYVNDLGNPKGTEYARPVGSSVYPFRGR | 242 |
| VvLOX | RHVFVONWYPAKIKTKRHFVFNKTYLSEFPLPRVYRFEELHHRGDL...TGLEKEDRVYDYVNDLGNPKDRDLKYARPVGSSVYPFRGR | 232 |
| PdLOX | RHVFVONWYPAKIKTKRHFVFNKTYLSEFPLPRVYRFEELHHRGDL...KGLQEWFRVYDYVNDLGNPKGSKYARPVGSSVYPFRGR | 236 |
| AtLOX6 | TILPANTVHHSKNDMPQRHFRSQPLPSFTPDGKELRKLKLSRGG...KGRKPHFRVYDYVNDLGNPKDGRM.TERVVPLPFRGR | 288 |
| MdLOX8b | TILPANTVHHSKNDLESRLFRKQACPGGTPPGKDLRRELLSRGG...KGRKPHFRVYDYVNDLGNPKSKDLARPVGGE.FRYPFRGR | 293 |
| | | |
| PcLOX1 | TGRPTKIDPNTSSRLPLQSLDIYVPRDEFHLKLSDFLALYKSLAQFTKPELESFDQTPSEFDSFEDVLIKVKGGIPLPEG.LLRDGDNIAPAM | 335 |
| PcLOX7 | TGRPATKIDPNTSSRLPLQSLDIYVPRDEFHPKMSDILAVYKSLPQVLKPEIRDLVGNKDFESMEVLIKVEGGLEPDG.LIKHSIDSTPEM | 341 |
| AtLOX1 | TGRPKIDPNTSSRLPTSSLDIYVPRDEFHLKMSDFLALYKSLAQFQALEAVFDITPKFESFEDVLIKVEGGIDLNQALDSVKNILDM | 332 |
| MdLOX7a | TGRPATKIDPNTSSRLPLQSLDIYVPRDEFHPKMSDILAVYKSLPQVLKPEIRDLVGNKDFESMEVLIKVEGGLEPDG.LIKHSIDSTPEM | 341 |
| VvLOX | TGRPSEKIDPNTSSRLPLVMSLDIYVPRDEFHLKMSDFLALYKSLVQFLPEFEALCDITPNEFDSFQVDLIKVEGGIKVEGPEGLDKNDIPLM | 332 |
| PdLOX | TGRPATKIDPNTSSRLPLMSLDIYVPRDEFHLKLSDFLALYKSLVQFIRPELEALFDITPNEFDSFEDVLIKVEGGIPLPEG.LLRDGDNIAPAM | 335 |
| AtLOX6 | TGRPLYSKDPQSSNG...KEKEEYVYPRDEFHLKRDTRAGRKAIFNLYSIAAALSNDIPITCSIDNLYKSNLYG.HTEPDTGLGGFTGG | 385 |
| MdLOX8b | TGRPTKIDPNTSSRL...EKPHVYVYPRDEFHLKQNTSTGRKALLNLSIAAATLSSIDNPECHSDDIYSDVLRKEDDFEKGKLFIS | 391 |
| | | |
| PcLOX1 | LKELFRIDGQALRFPFPVINDSAWRDEFERMLAGNPVNIARQVPIASRDLDPKYGDANSITHEHKNND...GLTVDFALKKNLFLD | 433 |
| PcLOX7 | FKELFRIDGQALRFPFPVINDSAWRDEFERMLAGNPVNIARQVPIASRDLDPKYGDANSITHEHKNND...GLTIDEALMSNKLFLD | 439 |
| AtLOX1 | LKELFRIDGQALRFPFPVINDSAWRDEFERMLAGNPVNIARQVPIASRDLDPKYGDANSITHEHKNND...GLTVEALKEKRLFLD | 430 |
| MdLOX7a | FKELFRIDGQALRFPFPVINDSAWRDEFERMLAGNPVNIARQVPIASRDLDPKYGDANSITHEHKNND...GLTIDEALMSNKLFLD | 439 |
| VvLOX | LKELFRIDGQALRFPFPVINDSAWRDEFERMLAGNPVNIARQVPIASRDLDPKYGDANSITHEHKNND...DLTINAMEKRLFLD | 430 |
| PdLOX | LKELFRIDGQALRFPFPVINDSAWRDEFERMLAGNPVNIARQVPIASRDLDPKYGDANSITHEHKNND...GLTVEALKKNLFLD | 433 |
| AtLOX6 | FMNGLNVTITLLKVDTPAVTVDGFAWLRDEFERMLAGNPVNIARQVPIASRDLDPKYGDANSITHEHKNND...GLTVEALKEKRLFLD | 485 |
| MdLOX8b | MYKEVLSVFRWKLVEFAVINDFAWLRDEFERMLAGNPVNIARQVPIASRDLDPKYGDANSITHEHKNND...GMSVDRALKEKRLFLD | 489 |
| | | |
| PcLOX1 | HDLSLPLRRLNLSN...RKYSRTLFQNGTLKPIVIELSLP.PGQDFGCSVYVTAEGVEGSIWQAKAYALNDGHIQLSHWNHAA | 530 |
| PcLOX7 | HDWAPLRLNLSN...RAYSRTLFNNGTLPVIAHLSLPPGQDFGCSIKRYVTSQGVSSIWQAKAYALNDGHIQLSHWLRRAHAM | 536 |
| AtLOX1 | HDITLPLRRLNLSN...RKYSRTLFQNGTLKPIVIELSLP.PGQDFGAVSIVYVTEGVEGSIWQAKAYALNDGHIQLSHWQHAS | 526 |
| MdLOX7a | HDWAPLRLNLSN...RAYSRTLFNNGTLPVIAHLSLPPGQDFGCSIKRYVTSQGVSSIWQAKAYALNDGHIQLSHWLRRAHAM | 536 |
| VvLOX | HDVPLRRLNLSN...RKYSRTLFQNGTLKPIVIELSLP.PGQDFGAVNRYVTAEDVEGSIWQAKAYALNDGHIQLSHWNHAA | 527 |
| PdLOX | HDALPLRRLNLSN...RKYSRTLFQNGTLKPIVIELSLP.PGQDFGCSIKRYVTAEGVEGSIWQAKAYALNDGHIQLSHWNHAA | 530 |
| AtLOX6 | YHIDLPLVEKNSIKEDPKRYSRTLFYSKCALRPLATELSLPTAIS...ENKHYVTHGHDAITTHWLRKAKHNCSDAGHIQLNHWLRHAS | 582 |
| MdLOX8b | HDITPLRRLNLSN...RAYSRTLFVTPAIVRPLATELSLPTAISLSP.QNKRVTYHGHDAITTHWLRKAKHNCSDAGHIQLNHWLRHAS | 585 |
| | | |
| PcLOX1 | SEPVYIATNRQLSVHPVKLLPHRDTMNAVARQILNAGGLIETTVPFARYAMLSVYVYKDWNTEDALALIRGVAIKDKNSPHGRLLI | 629 |
| PcLOX7 | TEPVYIATNRQLSVHPVKLLPHRDTMNAVARQILNAGGLIETTVPFARYAMLSVYVYKDWNTEDALALIRGVAIKDKNSPHGRLLI | 635 |
| AtLOX1 | IEPVYIATNRQLSVHPVKLLPHRDTMNAVARQILNAGGLIETTVPFARYAMLSVYVYKDWNTEDALALIRGVAIKDKNSPHGRLLI | 626 |
| MdLOX7a | TEPVYIATNRQLSVHPVKLLPHRDTMNAVARQILNAGGLIETTVPFARYAMLSVYVYKDWNTEDALALIRGVAIKDKNSPHGRLLI | 635 |
| VvLOX | IEPVYIATNRQLSVHPVKLLPHRDTMNAVARQILNAGGLIETTVPFARYAMLSVYVYKDWNTEDALALIRGVAIKDKNSPHGRLLI | 626 |
| PdLOX | CEPVYIATNRQLSVHPVKLLPHRDTMNAVARQILNAGGLIETTVPFARYAMLSVYVYKDWNTEDALALIRGVAIKDKNSPHGRLLI | 629 |
| AtLOX6 | MEPVYIATNRQLSVHPVKLLPHRDTMNAVARQILNAGGLIETTVPFARYAMLSVYVYKDWNTEDALALIRGVAIKDKNSPHGRLLI | 682 |
| MdLOX8b | VEPVYIATNRQLSVHPVKLLPHRDTMNAVARQILNAGGLIETTVPFARYAMLSVYVYKDWNTEDALALIRGVAIKDKNSPHGRLLI | 685 |
| | | |
| PcLOX1 | EDYPYVADGLTWSAIEITVKEYCSFYVYDDIITQKDELQSWWELVREHGHDKDEPWPWRKLTRELVETITITWASLHAAVNGQYVYAGLP | 729 |
| PcLOX7 | EDYPYVADGLTWSAIEITVKEYCSFYVYDDIITQKDELQSWWELVREHGHDKDEPWPWRKLTRELVETITITWASLHAAVNGQYVYAGLP | 735 |
| AtLOX1 | KOYPYVADGLTWSAIEITVKEYCSFYVYDDIITQKDELQSWWELVREHGHDKDEPWPWRKLTRELVETITITWASLHAAVNGQYVYAGLP | 726 |
| MdLOX7a | EDYPYVADGLTWSAIEITVKEYCSFYVYDDIITQKDELQSWWELVREHGHDKDEPWPWRKLTRELVETITITWASLHAAVNGQYVYAGLP | 735 |
| VvLOX | DYYPYVADGLTWSAIEITVKEYCSFYVYDDIITQKDELQSWWELVREHGHDKDEPWPWRKLTRELVETITITWASLHAAVNGQYVYAGLP | 726 |
| PdLOX | EDYPYVADGLTWSAIEITVKEYCSFYVYDDIITQKDELQSWWELVREHGHDKDEPWPWRKLTRELVETITITWASLHAAVNGQYVYAGLP | 729 |
| AtLOX6 | DYYPYVADGLTWSAIEITVKEYCSFYVYDDIITQKDELQSWWELVREHGHDKDEPWPWRKLTRELVETITITWASLHAAVNGQYVYAGLP | 782 |
| MdLOX8b | EDYPYVADGLTWSAIEITVKEYCSFYVYDDIITQKDELQSWWELVREHGHDKDEPWPWRKLTRELVETITITWASLHAAVNGQYVYAGLP | 785 |
| | | |
| PcLOX1 | NRPLISRRFPEGTPYEELRTPKAFKLTAFAPLILLGMATVILSRPDELYLGRDTAE...WTHADIQSEDRKNEAFAKLEKNDDE | 827 |
| PcLOX7 | NRPLISRRFPEGTPYEELRTPKAFKLTAFAPLILLGMATVILSRPDELYLGRDTAE...WTHADIQSEDRKNEAFAKLEKNDDE | 833 |
| AtLOX1 | NRPLISRRFPEGTPYEELRTPKAFKLTAFAPLILLGMATVILSRPDELYLGRDTAE...WTHADIQSEDRKNEAFAKLEKNDDE | 824 |
| MdLOX7a | NRPLISRRFPEGTPYEELRTPKAFKLTAFAPLILLGMATVILSRPDELYLGRDTAE...WTHADIQSEDRKNEAFAKLEKNDDE | 833 |
| VvLOX | NRPLISRRFPEGTPYEELRTPKAFKLTAFAPLILLGMATVILSRPDELYLGRDTAE...WTHADIQSEDRKNEAFAKLEKNDDE | 827 |
| PdLOX | NRPLISRRFPEGTPYEELRTPKAFKLTAFAPLILLGMATVILSRPDELYLGRDTAE...WTHADIQSEDRKNEAFAKLEKNDDE | 827 |
| AtLOX6 | NRPLISRRFPEGTPYEELRTPKAFKLTAFAPLILLGMATVILSRPDELYLGRDTAE...WTHADIQSEDRKNEAFAKLEKNDDE | 882 |
| MdLOX8b | NRPLISRRFPEGTPYEELRTPKAFKLTAFAPLILLGMATVILSRPDELYLGRDTAE...WTHADIQSEDRKNEAFAKLEKNDDE | 885 |
| | | |
| PcLOX1 | KIKNRVGPVYVYLLPISGGGTLGKIPSSV | 862 |
| PcLOX7 | RKNRFGPAKIPYLLPISSEFGTLNKGPTSIN | 868 |
| AtLOX1 | TKNRIGLWVYVYLLPISSEGGYLRGIPSSV | 859 |
| MdLOX7a | RKNRFGPAKIPYLLPISSEFGTLNKGPTSIN | 868 |
| VvLOX | RKNRGPVYVYLLPISSEGGTLGKIPSSV | 859 |
| PdLOX | KIKNRVGPVYVYLLPISGGGTLGKIPSSV | 862 |
| AtLOX6 | KIKNRIGAGMPELLPISPIVYLRGIPSSV | 917 |
| MdLOX8b | RKNRSGAGTIPPELLPISPIVYLRGIPSSV | 920 |

Fig. 1: Sequence alignment of eight plant LOX proteins. Identical amino acid sequences are highlighted in black, and sequence similarities are shown in grey. Solid line represents PLAT domain, dotted line represents lipoxygenase domain, and asterisks represent putative iron binding sites. The accession numbers of plant lipoxygenase proteins in GDR or GenBank are: *Pyrus communis* PcLOX1 (PCP003542), *Pyrus communis* PcLOX7 (PCP003539), *Arabidopsis thaliana* AtLOX1 (NP_175900), *Malus domestica* MdLOX7a (AGI16416), *Vitis vinifera* VvLOX (NP_001268178), *Prunus dulcis* PdLOX (CAD10779), *Arabidopsis thaliana* AtLOX6 (CAG38328), and *Malus × domestica* MdLOX8b (AGK82795).

dopsis AtLOX1 (*Arabidopsis thaliana*), 69.7% and 96.8% with apple MdLOX7a (*Malus × domestica*), 88.1% and 69.8% with almond PdLOX (*Prunus dulcis*), 77.8% and 70.9% with grape VvLOX (*Vitis vinifera*), 43.3% and 40.8% with *Arabidopsis* AtLOX6, and 43.9% and 43.7% with MdLOX8a, respectively.

Phylogenetic analysis of *PcLOX1* and *PcLOX7*

Analysis of the phylogenetic relationship between *PcLOX1*, *PcLOX7* and other plant LOX was performed using MEGA 6.05 using the neighbor-joining method. *PcLOX1* and *PcLOX7*, together with MdLOX7a, PmLOX5, PpLOX2, PpLOX3, FaLOX, AdLOX5, DkLOX3, CaLOX1, MdLOX1c, FvLOX5, AdLOX2, DkLOX1, and AtLOX1 were grouped with 9-lipoxygenases and belong to the type 1 superfamily; while AtLOX6, MdLOX8b, and PbLOX6 were grouped with 13-lipoxygenases and belong to the type 2 superfamily (Fig. 2).

LOX activity and gene expression in fruit pulp during fruit ripening at ambient temperature

During ambient temperature storage, the respiration rate decreased on day 2, increased until day 8 and afterward decreased in control fruit, whereas it decreased before day 4 and was relatively stable afterward in 1-MCP-treated fruit (Fig. 3A). During the shelf life period, the respiration rate increased on day 1 of shelf life and then decreased in control fruit, while 1-MCP-treated fruit showed a similar pattern of change with much lower respiration rates (Fig. 3B).

In control fruit, firmness showed a rapid decrease after day 5. While in 1-MCP-treated fruit firmness decreased slowly, it remained higher than control after day 5 (Fig. 3C).

LOX activity showed a slight decrease before day 10 in control fruit

and then gradually increased to the highest value on day 20, whereas in 1-MCP-treated fruit, LOX activity decreased rapidly on day 3 and later increased slowly but was always lower than that of the control (Fig. 3D).

The expression patterns of *PcLOX1* and *PcLOX7* were similar to each other during storage. Their expression levels gradually increased during the entirety of the storage period in control fruit, while the expression pattern was similar but expression levels were much lower in 1-MCP-treated fruit (Fig. 3E, F).

LOX activity and gene expression in fruit peel during scald development

Scald was observed on day 120 of cold storage with a scald incidence of 56.4% in control fruit. Scald continued developing during shelf life of control fruit and maintained an incidence of 96.2% after 7 days of shelf life. In contrast, 1-MCP-treated fruit showed much lower scald incidence, with no scald during cold storage and an incidence of 12.72% after 7 days of shelf life (Fig. 4A).

In control fruit, LOX activity decreased on day 30 of cold storage and then increased gradually to its highest value during shelf life. While in 1-MCP-treated fruit LOX activity decreased on day 30, increased to its highest value on day 90 and decreased afterwards, LOX activity was lower than in control fruit at most time points except on day 90 and 120 (Fig. 4B).

The expression patterns of *PcLOX1* and *PcLOX7* were similar to each other during cold storage and shelf life. *PcLOX1* and *PcLOX7* expression gradually increased during cold storage and rapidly reached the highest level after 7 days of shelf life in control fruit, while 1-MCP treatment effectively inhibited their expression (Fig. 4C, D).

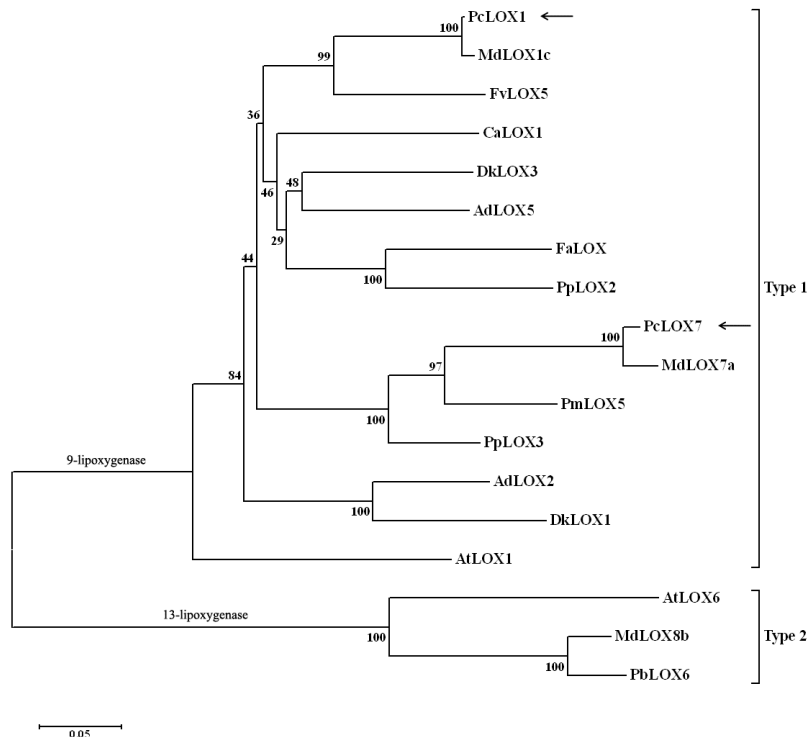


Fig. 2: Phylogenetic relationship of *PcLOXs* to other LOX proteins. The accession numbers of plant LOX proteins in GDR or GenBank are: *PcLOX1* (*Pyrus communis*, PCP003542), *PcLOX7* (*Pyrus communis*, PCP003539), MdLOX1c (*Malus × domestica*, AGK82778), MdLOX7a (AGI16416), MdLOX8b (AGK82795), PpLOX2 (ACH90245), PpLOX3 (*Prunus persica*, ACH91370), AdLOX2 (*Actinidia deliciosa*, ABF60002), AdLOX5 (ABF60001), FaLOX (*Fragaria × ananassa*, CAE17327), DkLOX1 (*Diospyros kaki*, AEZ50136), DkLOX3 (AEZ50135), CaLOX1 (*Capsicum annuum*, ACO57136), PmLOX5 (*Prunus mume*, XP_008245950), FvLOX5 (*Fragaria vesca*, XP_004302420), AtLOX1 (*Arabidopsis thaliana*, NP_175900), AtLOX6 (CAG38328), and PbLOX6 (*Pyrus × bretschneideri*, XP_009359326).

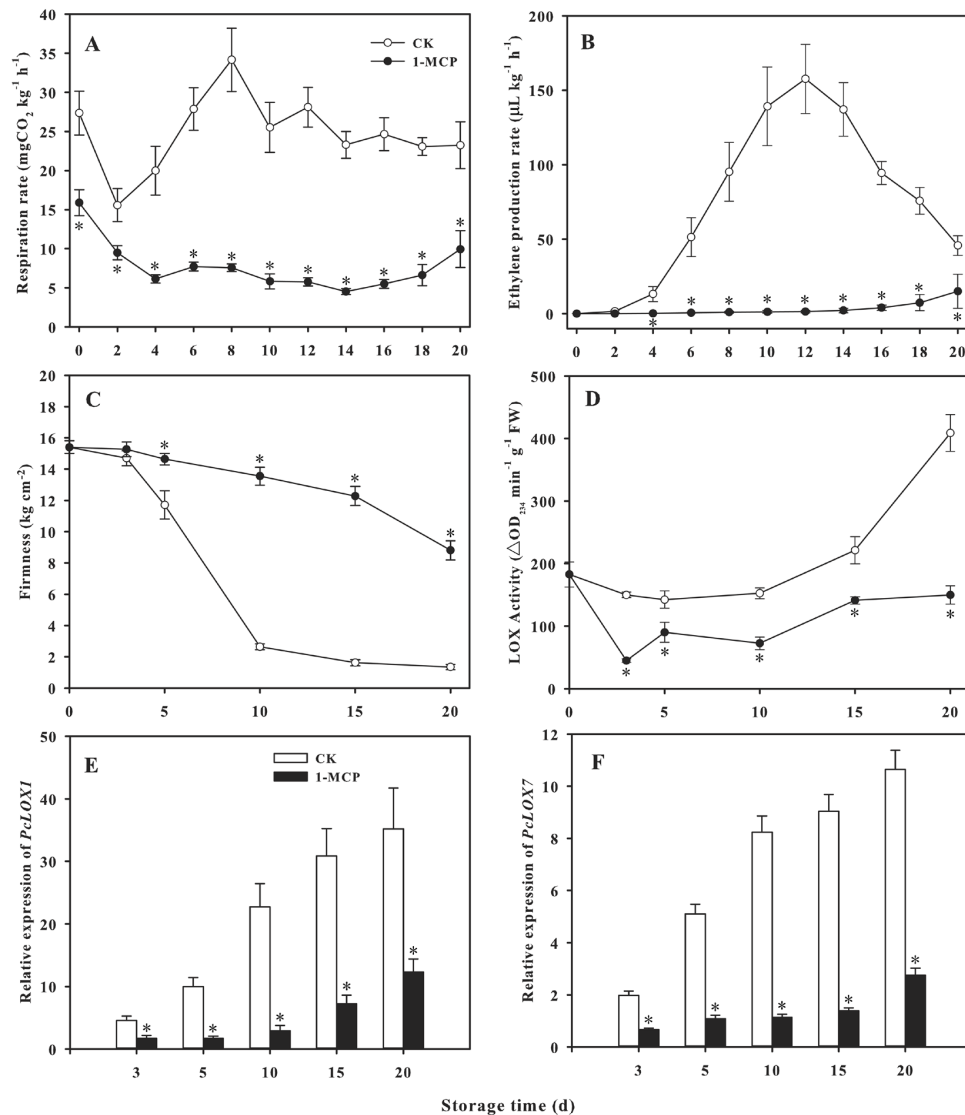


Fig. 3: LOX activity and gene expression in pulp during fruit softening in ambient temperature storage. (A) Respiration rate; (B) ethylene production rate; (C) firmness; (D) LOX activity; (E) and (F) expression of *PcLOX1* and *PcLOX7* genes. Data are the means \pm SE of three replicates. The vertical bars represent the standard errors of the means. The "*" represents a significant difference ($P < 0.05$).

LOX activity and gene expression during fruit ripening at shelf life

During ambient temperature storage, the ethylene production rate increased steadily in the control fruit until day 12 and then declined, whereas it was significantly inhibited by 1-MCP treatment (Fig. 5A). During the shelf life period, ethylene production rate increased significantly in the control until day 5, while it was almost completely inhibited in 1-MCP-treated fruit (Fig. 5B).

In control fruit, firmness showed a significant decrease during shelf life, while it had no significant change in 1-MCP-treated fruit (Fig. 5C).

In control fruit, LOX activity increased during shelf life, while in 1-MCP-treated fruit, it showed no significant change and much lower absolute activity levels than control (Fig. 5D).

The expression of *PcLOX1* decreased during shelf life in control fruit, while it showed a similar pattern with much lower levels in 1-MCP-treated fruit (Fig. 5E). The expression of *PcLOX7*, however, increased during shelf life in control fruit, while it was relatively stable with considerably lower levels in 1-MCP-treated fruit (Fig. 5F).

Discussion

'Wujiuxiang' pear (*Pyrus communis* L.) showed quick softening after several days at ambient temperature and is highly susceptible to superficial scald after a long incubation in cold storage, which can be considered a postharvest senescence, resulting in reduced fruit quality and economic loss (DONG et al., 2012; GAO et al., 2015; GUAN et al., 2014). Although it has been suggested that ethylene and LOX are involved in the fruit senescence process (FEYS et al., 1980; GAO et al., 2015; LI and WANG, 2009; LIU et al., 2013), little is known regarding the mechanism. In this study, respiration rate and ethylene production increased during senescence, yet they could be inhibited by 1-MCP treatment (Fig. 3A, B, C; 5A, B, C). In addition, LOX activity increased to its highest value in fruit pulp during late stages of ambient temperature storage and in peel and pulp at late stages of cold storage and shelf life (Fig. 3D; 4B; 5D), when pear fruit exhibited signs of softening and scald (Fig. 3C; 4A; 5C). These findings confirm the conclusion that ethylene and LOX activity are related to fruit ripening and scald development in pear (GAO et al., 2015; LI and WANG, 2009; LIU et al. 2013). The peak of ethylene

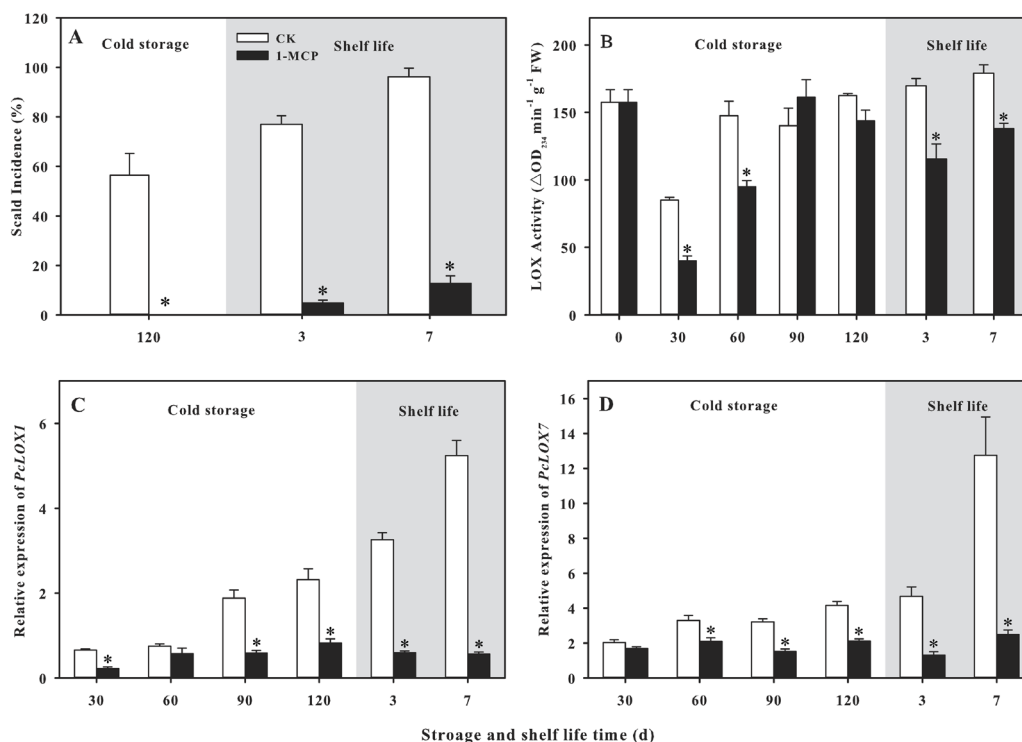


Fig. 4: LOX activity and gene expression in fruit peel during scald development during cold storage and shelf life. (A) Superficial scald incidence; (B) LOX activity; (C) and (D) expression of *PcLOX1* and *PcLOX7* genes. Data are the means \pm SE of three replicates. The vertical bars represent the standard errors of the means. The '*' represents a significant difference ($P < 0.05$), and the '**' represents a very significant difference ($P < 0.01$).

production was prior to the highest value of LOX activity (Fig. 3B, D; 5B, D), suggesting ethylene can directly regulate LOX activity, which is consistent with the results of other findings (GRIFFITHS et al., 1999; LI et al., 2006; SIRIKESORN et al., 2015).

Another interesting finding was that a significant decrease in LOX activity could be observed in control and 1-MCP-treated fruit at early stages of cold storage and in 1-MCP-treated fruit at early stages of ambient temperature storage (Fig. 3D, 4B). These findings suggest that low temperature and 1-MCP treatment can decrease LOX activity. It seems that low temperature and 1-MCP treatment could effectively inhibit ethylene production (Fig. 3B, 5B; LI and WANG, 2009; GAO et al., 2015), while ethylene production can directly regulate LOX activity (Fig. 3B, D; Fig. 5B, D; GRIFFITHS et al., 1999; LI et al., 2006; SIRIKESORN et al., 2015). However, the relationship between LOX activity and ethylene is complicated. SHENG et al. (2000) stated that LOX can regulate ethylene biosynthesis, while DIFILIPPI et al. (2005) argued that LOX activity may be independent of ethylene modulation. The different relationships observed between LOX and ethylene may be due to differences in plant species, cultivars or experiment designs, which need further study.

To explore which LOX gene members are involved in fruit ripening and scald development, two 9-LOX genes, *PcLOX1* and *PcLOX7* were characterized in *Pyrus Communis*. The deduced amino acid sequences of *PcLOX1* and *PcLOX7* each contain a conserved PLAT domain and a lipoxygenase domain with putative iron binding sites (Fig. 1), indicating the predicted protein sequences of *PcLOX1* and *PcLOX7* are accurate. The results of multiple sequence alignment and phylogenetic analysis indicated that *PcLOX1* and *PcLOX7* were highly conserved with other ripening- and senescence-related LOXs (Fig. 2), such as *MdLOX1* and *MdLOX7* in apple (VOGT et al., 2013), *PpLOX3* in peach (HAN et al., 2011), *DkLOX3* in persimmon (LV et al., 2014; MENG et al., 2016), and *AdLOX5* in kiwifruit (ZHANG et al., 2006; ZHANG et al., 2009). These analyses confirmed a high degree of LOX conservation during evolution and implied a function

for *PcLOX1* and *PcLOX7* in fruit ripening and plant senescence.

In most cases, expression of *PcLOX1* and *PcLOX7* increased during fruit ripening and scald development in 'Wujiuxiang' pear (Fig. 3E, F; 4C, D), consistent with the observed changes in LOX activity (Fig. 3D; 4B). The highest expression levels in pulp of EXP. 1 and in peel of EXP. 2 was observed at the end of the respective experiments, when pulp firmness was lowest and scald was most serious (Fig. 3E, F; 4C, D). These data suggest that *PcLOX1* and *PcLOX7* might be homologous genes involved in fruit ripening and scald development in 'Wujiuxiang' pear. However, while firmness decreased and LOX enzyme activity increased in fruit pulp at shelf life, *PcLOX1* expression declined significantly (Fig. 5D, E). One possible explanation of this result is that the mechanisms of fruit softening during ambient temperature storage and during shelf life after cold storage are different and may be associated with diverse LOX genes.

In conclusion, ethylene production increased during storage, contributing to higher expression levels of *PcLOX1*, *PcLOX7* and LOX enzyme activity, resulting in fruit ripening and scald development in 'Wujiuxiang' pear. In contrast, 1-MCP treatment could effectively suppress ethylene production, led to lower expression of *PcLOX1* and *PcLOX7* and lower LOX enzyme activity, resulting in slower fruit ripening and slower scald development.

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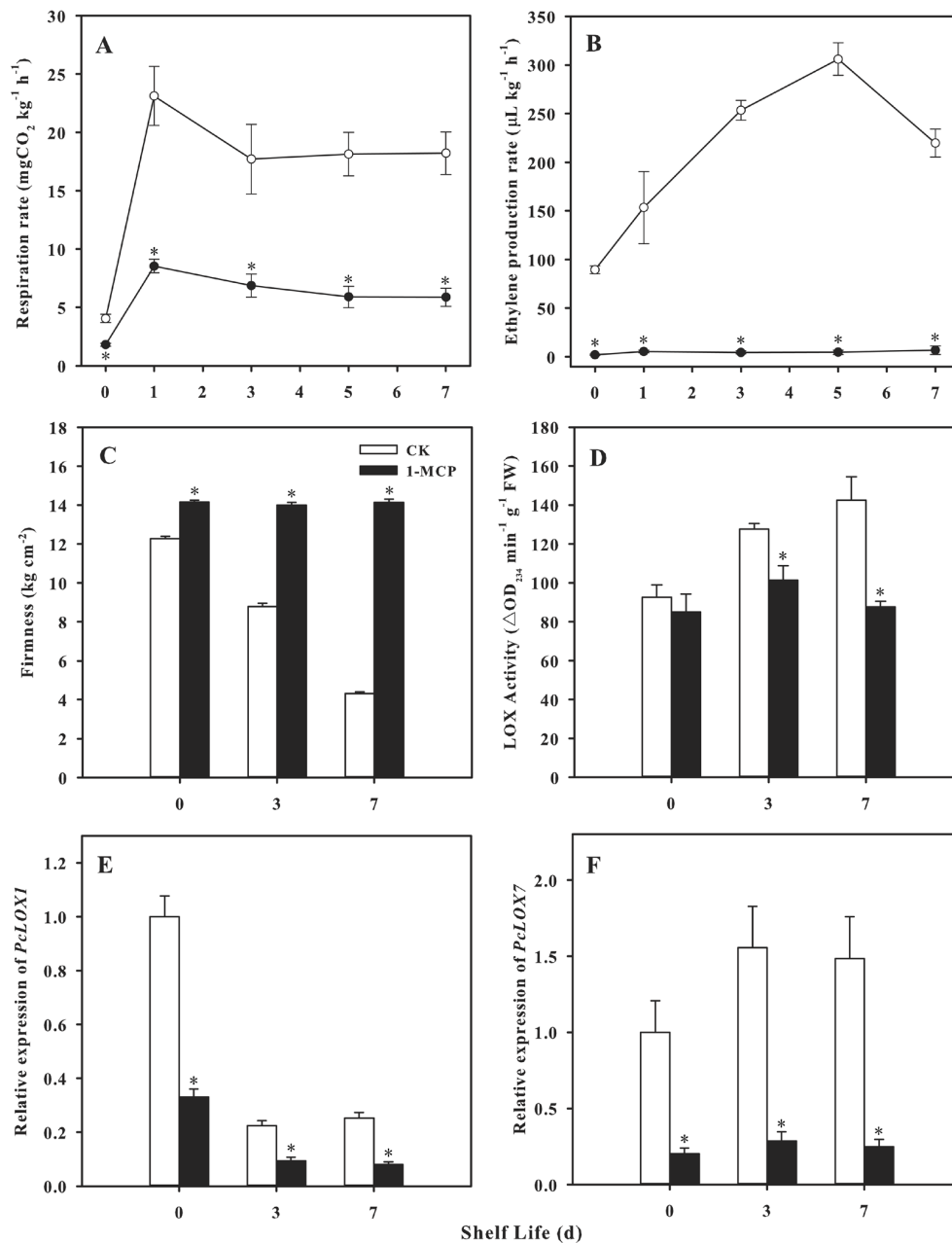


Fig. 5: LOX activity and gene expression in pulp during fruit softening at shelf life. (A) Respiration rate; (B) ethylene production rate; (C) firmness; (D) LOX activity; (E) and (F) expression of *PcLOX1* and *PcLOX7* genes. Data are the means \pm SE of three replicates. The vertical bars represent the standard errors of the means. The '*' represents a significant difference ($P < 0.05$).

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