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Divergent Expression of six Expansin Genes during Apple Fruit Ontogeny

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Summary

Six cDNAs encoding expansins were isolated from cultured shoots (*MdExp1*), the flesh of ripening fruit (*MdExp2*, *MdExp3*, *MdExp4*), and fruit pedicel tissue (*MdExp5*, *MdExp6*) of apple (*Malus* × *domestica* Borkh.). We report here the expression characteristics of these *MdExp* genes during fruit ontogeny. *MdExp1* was expressed in an up-down-up cycle from full-bloom stage to just before ripening. The expression of *MdExp2* and *MdExp5* was initiated when cell enlargement was started after the cell proliferation stage; the expression of *MdExp5* was limited to the early rapid growth period. The up-down-up cycles were also detected in the transcripts of *MdExp3* and *MdExp4* throughout both growth stages, but the expression of *MdExp3* was increased in the ripening stage. Transcripts of *MdExp6* were not detected in any growth stage, but were induced by wounding. These results suggest that apple fruit requires at least five expansin genes during ontogeny, and that *MdExp3* is a ripening-related expansin gene.

Zusammenfassung

Divergierende Expression von sechs Expansin-Genen während der Apfelfruchtontogenese. Sechs cDNAs zur Kodierung von Expansinen wurden aus kultivierten Sprossen (*MdExp1*), dem Fruchtfleisch reifender Früchte (*MdExp2*, *MdExp3*, *MdExp4*) und aus Fruchtstielgewebe (*MdExp5*, *MdExp6*) des Apfels (*Malus* × *domestica* Borkh.) isoliert. Wir berichten hier über das Expressionsverhalten dieser *MdExp*-Gene während der Fruchtontogenese. *MdExp1* wurde vom Stadium der Vollblüte bis kurz vor der Reife in einem Anstieg-Abfall-Anstieg-Zyklus exprimiert. Die Expression von *MdExp2* und *MdExp5* begann mit dem Zellwachstum im Anschluss an das Zellteilungsstadium; die Expression von *MdExp5* war begrenzt auf die erste Schnellwuchsphase. Ein Anstieg-Abfall-Anstieg-Zyklus wurde auch während beider Wachstumsphasen bei der Transkription von *MdExp3* und *MdExp4* beobachtet, doch erhöhte sich die Expression von *MdExp3* während des Reifestadiums. Transkripte von *MdExp6* wurden in keiner Wachstumsphase entdeckt, sie wurden jedoch bei Verwundung induziert. Diese Ergebnisse weisen darauf hin, dass bei der Apfelfrucht mindestens fünf Expansingene während der Ontogenese erforderlich sind und, dass *MdExp3* ein in Beziehung zur Reife stehendes Expansingen ist.

Key words. apple – expansin – *Malus* × *domestica* Borkh. – fruit development – fruit ripening – fruit ontogeny

Introduction

Expansins are cell wall 'loosening' proteins which are proposed to allow slippage between the cell wall network of cellulose microfibrils and adhering polysaccharide (MCQUEEN-MASON et al. 1992; COSGROVE 1993, 1998). Three families of expansins are now established, termed α- ,β-, and γ-expansins. In *Arabidopsis* the α-expansin family has 26 members, the β-expansin family has ten members, and the γ-expansin family consists of two members (LI et al. 2002). The presence of multigene families of expansins has been identified in many plant species (SHCHERBAN et al. 1995; CHO and KENDE 1997; COSGROVE et al. 1997; BRUMMELL et al. 1999), and their expression profiles suggest that different expansins play unique developmental or tissue-specific roles (MCQUEEN-MASON et al. 1992; LI et al. 1993; WU et al. 1996; CHO and KENDE 1997). Expansin genes are also expressed in developing and ripening fruits of tomato (ROSE et al. 1997; CATALA et al. 2000), strawberry (CIVELLO et al. 1999; HARRISON et al. 2001), and peach (HAYAMA et al. 2000). However, there is no report of apple fruit so far.

Apple fruits consist mainly of parenchymatous tissue forming the flesh, of which the cortex constitutes the greater proportion. Although minor growth occurs before blossom time, pollination stimulates the rapid development of the fruit (SMITH 1950). The growth is slow immediately after blossom time, gradually increases to a maximum, and then slows down again. This growth pattern results from two cell growth events. After the cell division period, cell enlargement begins and proceeds at a rapid rate. Furthermore, concurrent with cell enlargement, intercellular spaces, which at first are absent or very

small, increase to a constant maximum for the remainder of the season. Early in the cell enlargement phase, vacuoles form in the cells and increase in size as the cells enlarge, ultimately occupying most of the space in the centre of cells (SKENE 1966). The combined growth resulting from cell division, cell enlargement, and air space formation results in a general sigmoid curve when fruit volume or weight is plotted as a function of time (ABBOTT 1984). We are interested in clarifying whether or not different isoforms of expansin are involved in each growth stage.

The onset of apple fruit maturity is preceded by the production of ethylene in the tissues, which induces ripening of fruit and hastens abscission. Tomato, peach, and strawberry expansin genes upregulated during fruit ripening have been isolated (ROSE et al. 1997, 2000; CIVELLO et al. 1999; HAYAMA et al. 2000). Furthermore, repression of a fruit-ripening-specific expansin (*LeExp1*) in tomato resulted in reduced softening, and over-expression of *LeExp1* resulted in enhanced softening (BRUMMELL et al. 1999). These results suggest that, in addition to the activity of fruit cell wall hydrolases such as polygalacturonase and cellulase, expansin has an important function during ripening.

To characterize the expansin genes related to apple fruit ontogeny, we analysed the expression profiles of six expansin genes, which were cloned from cultured shoots and fruit. The results show that each expansin gene was expressed with a unique profile pattern. We consider one of them to be a ripening-related expansin gene.

Materials and Methods

Plant materials

Samples were collected at the experimental farms of Hirosaki University and the Aomori Apple Experimental Station. In 2001, apple (*Malus* × *domestica* Borkh.; unless otherwise stated, cv. 'Golden Delicious') fruit were harvested just before ripening (11 October) and placed in a 24 ± 1 °C room equipped with a ventilation system. In 2002, the fruits were sampled weekly from full-bloom stage (6 May) to early ripening stage (4 November). As samples, we used the whole of the receptacle tissue until 5 June, and thereafter the cortex tissue prepared by cutting with a kitchen knife. Shoot tissue (cv. 'Orin', 3 cm long) was obtained from an *in vitro* cultured plantlet that was originally derived from a stem tip and subcultured every four weeks on MS medium (1× MS basal medium, 1 mg L^{-1} benzylaminopurine, 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, 8 g L^{-1} agar, pH 5.7) for 3 weeks at 24 °C (16 h light/8 h dark). Fruit pedicel tissue was obtained from 65 mature fruits (18 October 2001) from a progeny of 'Megumi' × 'Toko' (strain No. 94-64). Immediately after harvesting, we cut about 2 mm of pedicel tissue, encompassing the abscission layer, from the tip with a razor blade. All tissues were frozen in liquid N_2 and then stored at –80 °C until extraction of nucleic acids.

RNA extraction

Total RNA was extracted from tissues as described by HARADA et al. (2000).

Cloning of expansin cDNA

RT-PCR was performed on 3 μg of total RNA, from the flesh of fruit ripened at 24 °C for six days and from the pedicel tissue described above, by using a Gene Amp RNA PCR Kit (Perkin-Elmer). Degenerate primers (MdExpF, 5′-GSNCAYGCNACNTTYTAYG-GNG-3′; MdExpR, 5′-YTGCCARTTYTGNCCCCA-RTT-3′) were designed from a conserved expansin

Table 1. Primer sets used for the PCR amplifications of gene-specific probes.

Gene name	Primer name	Primer sequence	Accession no. (nucleotide)
MdExp1	MdExp1F	5' TCAAGGTCACAACCAGTGA 3'	AB099925 (566–1022)
	MdExp1R	5' GTCAAAGTCCACAACCTCTG 3'	
MdExp2	MdExp2F	5' GGCAAGCAATGTCAAGAAACTG 3'	AB099926 (470–1085)
	MdExp2R	5' TTAAAGGCGCAGAATAAGCAGC 3'	
MdExp3	MdExp3F	5' GAGCAACTCTTACCTCAACG 3'	AB099927 (531–823)
	MdExp3R	5' GCGGGTGCCAGTTAATTAG 3'	
MdExp4	MdExp4F	5' GAGCAACTCATACCTCAATG 3'	AB099928 (531–881)
	MdExp4R	5' CATTAAGGTTACAATTGAG 3'	
MdExp5	MdExp5F	5' TTTGCTTCAAAGTGAGTGACC 3'	AB099929 (779–980)
	MdExp5R	5' CCTCATGAATTAACAGCGGTG 3'	
MdExp6	MdExp6F	5' AACATCGTCCCTTCCAACTG 3'	AB099930 (613–888)
	MdExp6R	5' CATGGCCAGATAACCAAAGC 3'	
MdACS5	MdACS5F	5' ACATCGAATGCAACCGCAACCTTA 3'	AB034992 (862–1545)
	MdACS5R	5' TCTAAGTGGCTCGAACAAGAGG 3'	
MdH1	MdH1F	5' TGGCGACCGAAGAAGCCACC 3'	AB099931 (1–431)
	MdH1R	5' CGGCTTCTTCTTCTTCACCGG 3'	
MdACO1	MdACO1F	5' TGAAATTCCAAGCCAAGGAG 3'	AF030859 (3558–3780)
	MdACO1R	5' TTCAACTACACAAACAGTGG 3'	
MdPG	MdPGF	5' AAAGGTCATGGAATTGATCAGGCC 3'	L27743 (433–1650)
	MdPGR	5' ATGCCCATAATTATGAACCC 3'	

amino acid sequence in tomato (ROSE et al. 1997). Each fragment cloned into the pBluescript KS vector (Stratagene) by using the TA cloning method (KOVALIC et al. 1991) was sequenced, and then the 3' region containing noncoding sequence was also determined by using the 3′ RACE method (CHENCHIK et al. 1996).

Southern and Northern blot analyses

Nucleic acid extraction and gel blot analyses were performed as described by SUNAKO et al. (1999). The probe for each gene was prepared by PCR using a cDNA clone as a template. The sequences of the oligonucleotide primers used are shown in Table 1

Measurement of ethylene production rates and fruit firmness

To determine the rate of ethylene production, we enclosed three intact fruits in a gas-tight container (2.6 L) equipped with septa, then sampled 1 mL of gas from the container headspace through a syringe. The ethylene concentration in the sample was measured with a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionisation detector. The fruit firmness was measured with a Fruit Pressure Tester (FT-327; Facchini, Milan, Italy) fitted with an 11-mm-diameter probe. Two skin discs (~2.5 cm in diameter) were removed from opposite sides of each fruit. The penetrometer probe was pressed into the tissue of the cut surface to a depth of 8 to 9 mm in a single smooth motion. Five fruits per sample were used for measurements.

Elicitor treatment and wounding of cultured shoot

Partially purified elicitor was prepared from yeast extract (OETIKER et al. 1997). Cultured shoots (cv. 'Orin'), weighing 1 g each, were incubated for 3 h at 24 °C in sterilized water with the elicitor (200 μg mL^{-1}) in an Erlenmeyer flask (100 mL) with shaking (100 rpm). For wounded tissue, the cultured shoot was cut into small pieces (about 5 mm square) with a razor blade, and the pieces were incubated for 3 h at 24 °C in a test tube. These samples were frozen in liquid N_2 immediately after treatment and then stored at –80 °C until extraction of RNA.

Results and Discussion

Molecular characteristics of six expansin homologues

We cloned three expansin homologues from the flesh of ripening fruit and two from the fruit pedicel. Fig. 1

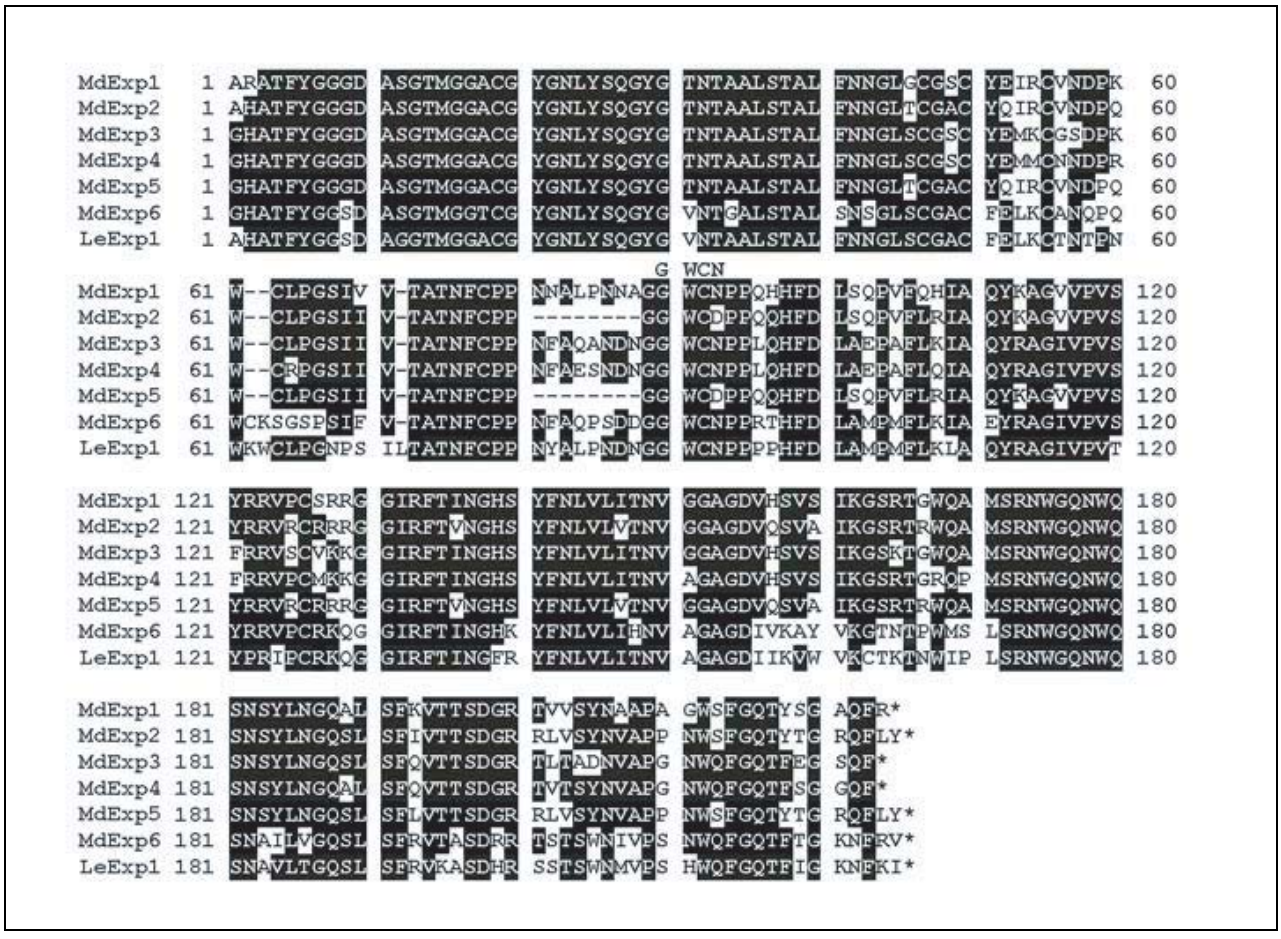


Fig. 1. Alignment of deduced amino acid sequence of expansin without signal peptide in apple (*MdExp1–6*) and tomato (*LeExp1*). GWCN motif is indicated.

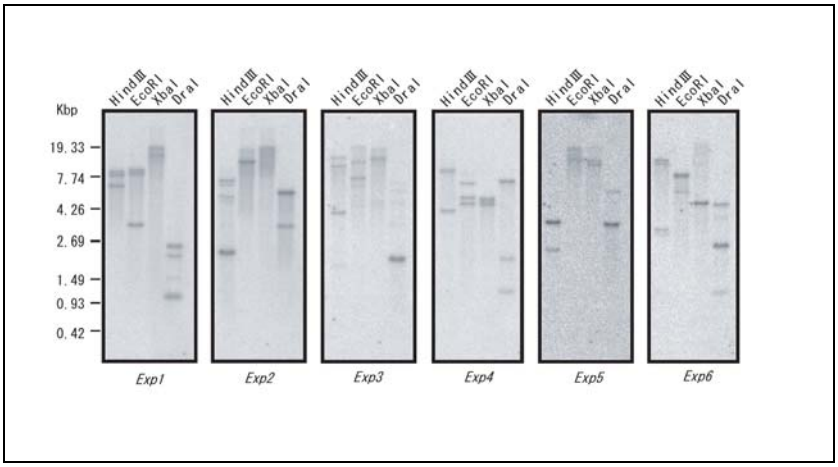


Fig. 2. DNA gel blot analyses showing the specificity of the probes used. Ten micrograms of genomic DNA (cv. 'Golden Delicious') was digested with *Hin*dIII, *Eco*RI, *Xba*I, and *Dra*I. Digested DNA was electrophoresed, blotted onto a nylon membrane, and hybridised with each *MdExp* probe.

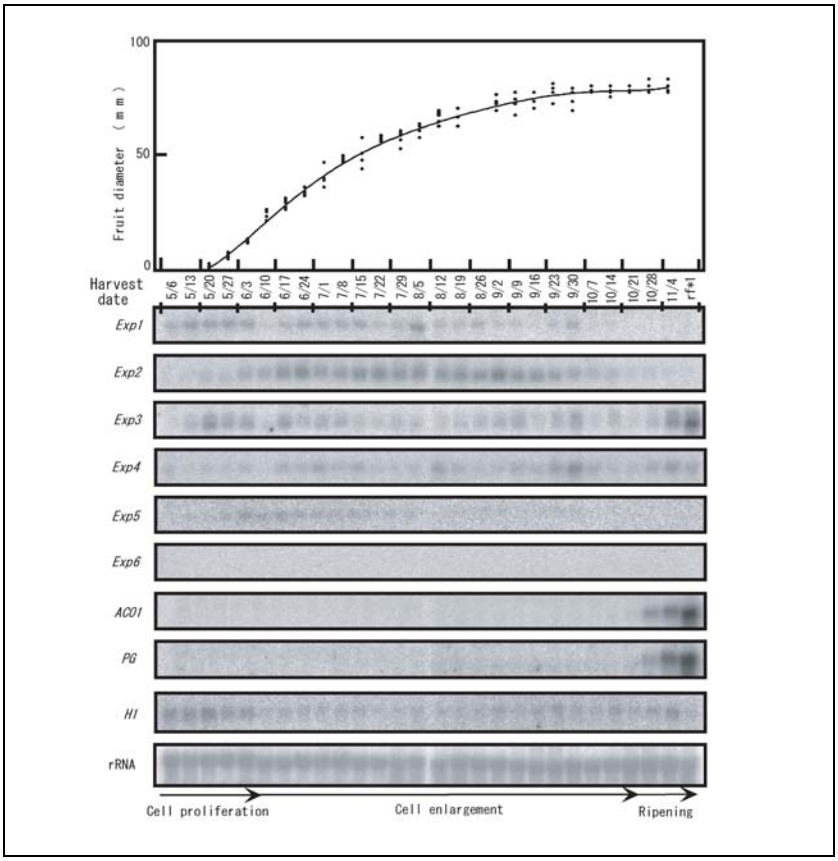


Fig. 3. Changes in fruit equatorial diameter and *MdExp* expressions during fruit ontogeny. The expressions of the ripening-related genes (*ACO1*, *PG*) and of the cell-division-related gene (*H1*) were also assayed. *RNA used in this lane comes from fruit that were incubated at 24 °C for 6 days.

shows the alignment of the putative amino acid sequences, which lack the sequences of the 5′ untranslated and signal peptide regions, of the five apple expansin homologues and *MdExp1*, which we cloned from a cultured shoot (unpublished data). The identity of the deduced amino acid sequences with the ripening-related expansin gene in tomato (*LeExp1*, accession number U82123) was 51.7% to 67.5%. Furthermore, these homologues conserved the α-insertion containing GWCN residues, which are considered to be characteristics of α-expansin (Li et al. 2002). Therefore, we designated them *MdExp2–6* (accession number AB099925–AB099930). PCR products that amplified the 3′ region (mainly untranslated region) of each *MdExp* were prepared for gene specific probes. The Southern hybridisation signal patterns in Fig. 2 revealed that each *MdExp* probe worked as a gene-specific probe. Although *MdExp1*, *MdExp2* and *MdExp4* produced two or more hybridised bands, these results may be due to the heterozygosity characteristic of apple genome or be indication for presence of several closely related genes.

Expression of MdExp during fruit ontogeny

The primer sets used for the PCR amplifications are given in Table 1. The length of the hybridisation probes was between 201 bp for *MdExp5* and 615 bp

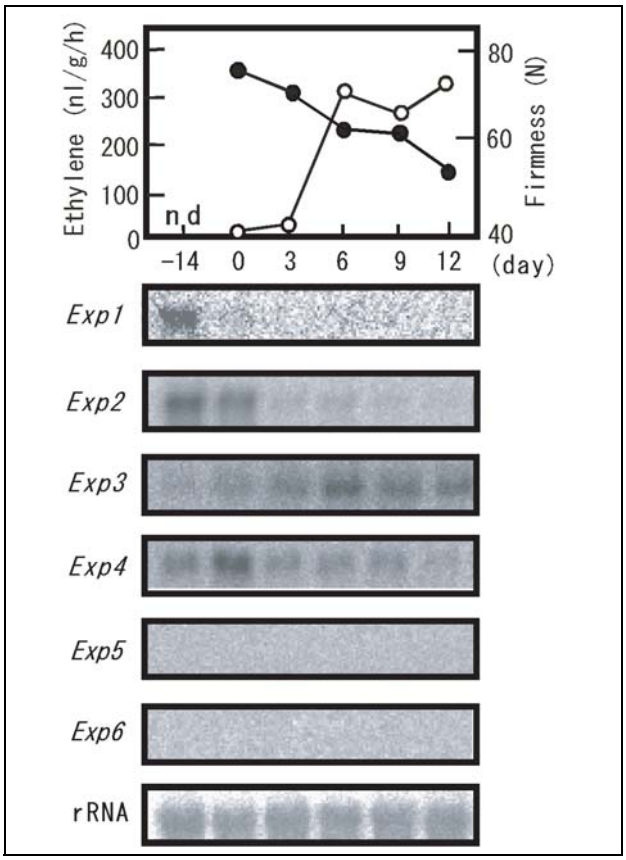


Fig. 4. Changes in the expression of *MdExp* genes during fruit ripening. Commercial harvest stage (11 Oct) is shown as day 0. The harvested fruit were kept for 3 to 12 days at 24 °C. After measurement of ethylene production rate (❍) and fruit firmness (●), total RNA was extracted every 3 days. Fruit attached to the tree on 27 Sep is indicated as –14.

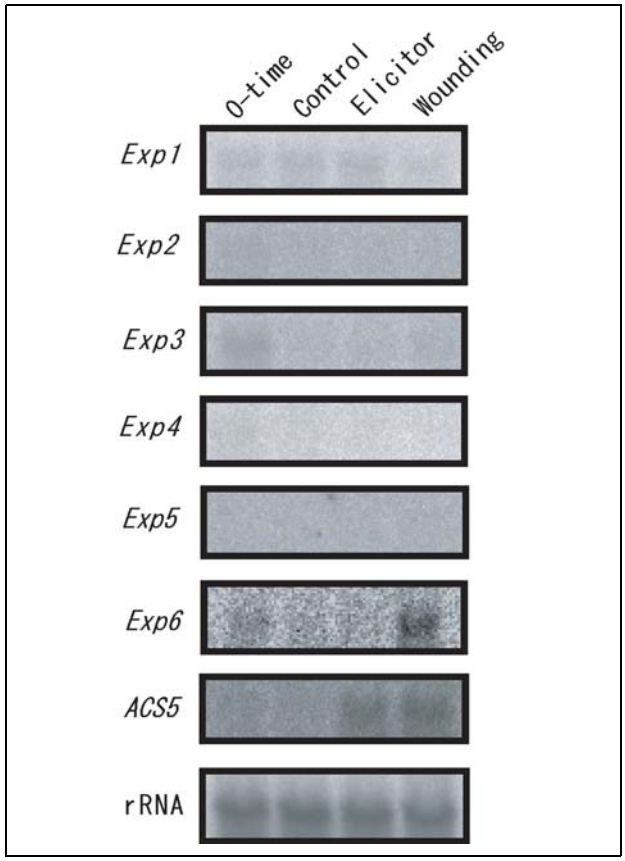


Fig. 5. Effect of elicitor treatment and wounding on *MdExp* gene expression in a cultured shoot. *MdACS5* was also used as a wound-responding gene. Each treatment was performed for 3 h.

for *MdExp2*. To see the expression pattern of each *MdExp* gene during fruit ontogeny, we used RNA fractions extracted weekly from fruits from the full blossom to ripening stages in Northern blot analysis (Fig. 3). We used a cell-division-related gene (*H1*: histone 1, cloned by us) and two ripening-related genes (*ACO1*: ACC oxidase, ROSS et al. 1992; *PG*: polygalacturonase, ATKINSON 1994) as marker genes to show the developmental phase. *MdExp1* expression showed a repeated up and down pattern throughout fruit development. *MdExp2* transcript was detected during the cell enlargement phase after the cell proliferation phase. The expression of both genes ceased during the ripening phase. On the other hand, *MdExp3* and *MdExp4* showed an up and down cycle expression pattern throughout the ontogeny. It seems that the blot for *MdExp3* shows two bands indicating the presence of transcript from closely related gene. Another explanation could be that the additive signal is a transcript from utilization of different transcription start-site or poly(A) signal of the *MdExp3*. The expression of *MdExp5* was detected only early in the cell enlargement phase. *MdExp6* expression was not detected at any time.

Apple fruit development from the pollination to the ripening stage can be divided broadly into three phases (SMITH 1950). The first period is about 4 weeks after pollination, in which growth by cell proliferation occurs. This phase was characterized by the relatively high expression of *H1* (Fig. 3). The expression of *MdExp1* and *MdExp3* was detected in this first phase, but their transcripts were also present in the following cell enlargement phase. *MdExp2* was abundant only in the second growth phase and may be of use in identifying this phase. Although the expression of *MdExp5* was not abundant, its transcription coincided with the extremely rapid growth period in June, suggesting its intimate involvement in this growth stage. Only *MdExp3* expression increased in the ripening stage, especially at full ripeness. Therefore, independent expression patterns of five expansin genes were detected in Northern analysis of *MdExp* genes during fruit ontogeny.

MdExp3 is a ripening-related expansin gene

We investigated the expression of the six *MdExp* genes in the flesh of ripening fruit, whose firmness decreased rapidly in inverse proportion to the rate of ethylene production (Fig. 4). *MdExp1* and *MdExp2* transcripts diminished after plunging into ripening. *MdExp4* expression was temporarily increased at commercial harvest time then decreased at the ripening stage. On the

other hand, no mRNAs of *MdExp5* or *MdExp6* were detected before or after ripening. Only *MdExp3* was predominantly expressed almost in parallel with ethylene production rate during ripening. Furthermore, treatment of immature fruit with 1-methylcyclopropene (an antagonist of ethylene action) almost perfectly suppressed the induction of *MdExp3* expression (data not shown), suggesting that *MdExp3* is regulated by ethylene. These expression features indicate that *MdExp3* is a ripening-related expansin gene in apple. So far, ripening-specific expansin genes have been reported in tomato (*LeExp1*, ROSE et al. 1997), peach (*PchExp1*, HAYAMA et al. 2000), and strawberry (*FaExp2*, CIVELLO et al. 1999; HARRISON et al. 2001). *MdExp3* shares 55.7 % amino acid identity with *LeExp1*, 89.0 % with *PchExp1*, and 90.9 % with *FaExp2*. However, the flesh of these fruits develop from morphologically different organs.

Wounding-induced expansin gene

Although no *MdExp6* mRNA was detected in any tissues we investigated (Figs. 3, 4), the fruit pedicel tissues containing the abscission layer expressed it at fruit maturity stage (data not shown). The natural abscission process can be interpreted as a self-inflicted wound. Furthermore, wound-induced expansin expression was observed in rice internodes (LEE and KENDE 2001, 2002). Therefore, we investigated the expression of *MdExp* genes by wounding. As shown in Fig. 5, only *MdExp6* expression was induced by wounding, while *MdExp1* and *MdExp2* expression, if anything, appeared to decrease. On the other hand, treatment with the elicitor did not induce the expression of any of the six *MdExp* genes. Although the expression of many wound-inducible genes (CHEONG et al. 2002) can be also induced by elicitors, as with *MdACS5* (wound-responsive gene encoding ACC synthase in apple; SUNAKO et al. 2000) in Fig. 5, we observed different expression profiles of *MdExp6* between treatments. This result suggests that the role of *MdExp6* lies in cell wall repair when tissue is wounded by external factors (LEE and KENDE 2001).

We are currently studying the relationship between *MdExp3* expression levels and apple fruit storage ability. Preliminary data indicate that cultivars with long storage ability are likely to show low levels of *MdExp3* expression during ripening. Transgenic tomato in which the expression of *LeExp1* was suppressed remained firm longer than wild-type fruit during ripening (BRUMMELL et al. 1999). We are also trying to develop a transgenic apple plant with reduced *MdExp3* expression during ripening.

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