

Isolation of a cDNA Encoding a 31-kDa, Pathogenesis-related 5/thaumatin-like (PR5/TL) Protein Abundantly Expressed in Apple Fruit (*Malus domestica* cv. Fuji)

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A fruit-specific and pathogenesis-related 5/thaumatin-like (PR5/TL), 31-kDa protein was isolated by 2D-PAGE from fully-grown apples (*Malus domestica* cv. Fuji) and named Mdt11 (*Malus domestica* thaumatin-like protein 1). Using the N-terminal sequence of the protein, the full-length cDNA encoding Mdt11 was isolated. The cDNA clone (*Mdt11*) consists of 944 bp with an open reading frame (ORF) of 744 bp encoding a protein of 247 amino acids. The deduced amino acid sequence of *Mdt11* shows high similarity to the sequences of PR5/TL proteins. Mdt11 is a slightly acidic protein with a putative signal peptide and a putative N-glycosylation site, and lacks a C-terminal extension. This suggests that Mdt11 is an apoplastic glycoprotein. Results of northern blotting indicated that expressions of *Mdt11* are developmentally regulated. Southern blot analysis showed that *Mdt11* may be present as a single copy, and there exist other genes closely related to *Mdt11* in the apple genome.

Key words: thaumatin-like; pathogenesis-related; apple (*Malus domestica* cv. Fuji); fruit-specific; Mdt11

Fruits are different from other plant tissues in many respects. The cells in most fruits undergo rapid expansion in the course of fruit growth and accumulate a large amount of sugars and starch.¹⁾ Hence, fruits become strong sinks of metabolites in plants. As fruits ripen, significant changes occur in their structure, physiology, and chemical constituents. All these characteristics of fruits are related to different expressions of various fruit-specific proteins. Proteins are very important in this aspect,

although they are generally present in low concentrations in fruits.

Apple (*Malus domestica*) accounts for nearly 60% of world temperate fruit tree production; hence, its physiology and biochemistry are better known than those of other fruits.^{2,3)} Studies in apple fruits have been focused mainly on the proteins and enzymes related to the ripening process. Ethylene-forming enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) oxidase and ACC synthase were studied extensively.⁴⁻⁸⁾ Other enzymes involved in the degradation of the cell wall such as beta-galactosidase were isolated in apple fruits.⁹⁾ NADP-dependent sorbitol dehydrogenase,¹⁰⁾ sorbitol-6-phosphate dehydrogenase,¹¹⁾ and polyphenol oxidase^{12,13)} were also characterized in relation with the metabolism of carbohydrates and phenolic compounds.

Because of their high content of carbohydrates and other nutrients, fruits are susceptible to various infections. Recently, some proteins involved in defensive roles were reported in apple fruits. A gene encoding the polygalacturonase inhibitor was found to be regulated in the course of fruit development and also activated by wounding and fungal infection.¹⁴⁾ Some cDNA clones related to defensive roles, such as cysteine protease inhibitor and lipoxygenase, were shown to be expressed in young fruits and mature flowers by an analysis of expressed sequence tags.¹⁵⁾ Recently, a 60-kDa protein was reported to be abundant in mature apple fruits.¹⁶⁾

In this study, we analyzed the apple fruit-specific proteins for the purpose of improving fruit qualities and characterized a 31-kDa protein as one of the major protein constituents of a mature apple fruit.

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Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; bp, base pair(s); IEF, isoelectric focusing; kDa, kilodalton; Mdt11, *Malus domestica* thaumatin-like protein 1; ORF, open reading frame; RT-PCR, reverse transcription and polymerase chain reaction; PR5/TL, pathogenesis-related 5/thaumatin-like; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UTR, untranslated region; WAP, weeks after pollination

Material and Methods

Plant materials. Apple fruits (*Malus domestica* cv. Fuji) in various developmental stages were harvested from the orchard of Chungnam National University Agricultural College, Taejon, Korea. The fruit flesh was frozen in liquid nitrogen and stored at -70°C . Leaves (fully expanded) were also collected and stored as the same manner.

Two-dimensional gel electrophoresis and analysis of N-terminal amino acid sequences. Fully-grown fruits at 14 weeks after pollination (WAP) and fully expanded leaves were used for protein extraction. The extraction methods were followed as described in Hurkman and Tanaka.¹⁷⁾ A total of 10 μg of protein from the leaf and the fruit flesh was analyzed by isoelectric focusing (IEF) in the first dimension, followed by a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The IEF gel for the first dimension contained 1.6% 5/7 ampholyte and 0.4% 3/10 ampholyte, and 15% acrylamide gels were used for the SDS-PAGE. Gel slices containing fruit-specific protein spots were excised directly from the 2D-PAGE gel and then embedded on a SDS-PAGE gel to concentrate them. After electrophoresis, the collected protein was electroblotted onto a polyvinylidene difluoride membrane. An automated Edman amino acid sequencing of the protein was then done using the Beckman PI-2090 liquid-pulse sequencer.

RNA isolation, reverse transcription, and PCR. The total RNA from apple fruits and leaves was extracted with an Extract-A-Plant RNA isolation kit (Clontech). Poly(A)⁺ RNA was isolated from the total RNA with a Poly-A-Tract mRNA isolation kit (Promega). First strand cDNA was synthesized from Poly(A)⁺ RNA by SuperScript II reverse transcription system (Gibco-BRL) with random hexamers as primers. PCR was done with the synthesized cDNA under the following conditions: 30 seconds at 94°C , 30 seconds at 53°C and 30 seconds at 74°C (33 cycles). For the PCR reaction, a forward primer [5'-TT(C/T)GG(A/G/C/T)CA(A/G)AA(A/G)CC(A/G/C/T)CA(A/G)-3'] was deduced from the known N-terminal sequence of the 31-kDa protein and a reverse primer [5'-TAGCT(A/G)TA(A/G)GC(A/G)TC(A/G)GG(A/G)CA-3'] was constructed from the conserved region of the previously published nucleotide sequences of PR5/TL genes.¹⁸⁻²⁰⁾ The amplified cDNA fragment was cloned into the pGEM-T-Easy vector (Promega) for sequence analysis and used as a probe in a cDNA library screening as described below.

Cloning of *Mdt11* cDNA. An apple fruit cDNA

library was constructed with a lambda ZAP-cDNA GigapackII Gold cloning kit (Stratagene), as described in the manufacturer's instructions. Approximately 5×10^5 recombinant lambda phages were screened with the cDNA probe obtained by RT-PCR as above, which was labeled with [α - ^{32}P]dCTP. The labeling reaction was done with a Prime-a-gene labeling system (Promega). Hybridization was done at 42°C and washed as described in Sambrook *et al.*²¹⁾ Isolated phage clones were converted to pBluescript derivatives, as in the manufacturer's instruction (Stratagene). Nucleotide sequencing was done using the Sequenase 2.0 kit (US Biochemical) in both orientations with several custom-made oligonucleotide primers (Genotech, Korea).

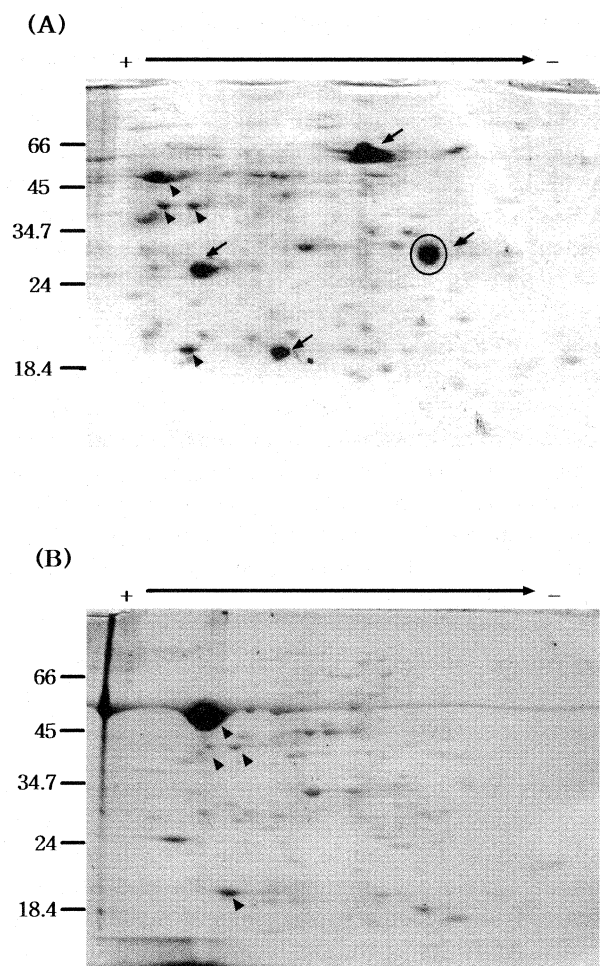


Fig. 1. Comparison of the Protein Constituents in Apple Fruit and Leaf.

Total proteins (10 μg) were extracted from the flesh of a fully-grown fruit at 14 WAP (A) or from the leaf tissue (B), separated by 2D-PAGE, and stained with Coomassie Brilliant Blue. IEF gels of the first dimension (horizontal) contained 1.6% 5/8 ampholyte and 0.4% 3/10 ampholyte, and 15% acrylamide gels were used in the SDS-PAGE of the second dimension (vertical). Fruit-specific proteins are marked with arrows and the 31-kDa protein with a circle. Triangles indicate the proteins that are found in both tissues. Molecular mass standards are shown in kDa.

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ttcggcacgaggcaaacaggcaattaagacatatcaATGTCGATGATGAAGAGCCAAGT 60
                                     M A M M K S Q V
AGCTCCTCGGCCTACCTTGGCCATCCTCTTCTTCTCAGGTGCACATGCA GCGAAAAAT 120
9  A P R P T L A I L F F F S G A H A A K I
CACTTTCACA AACAACCTGCCCAACACTGTCTGGCCAGGAACCTTAACCGGTGACCAAAA 180
29  T F T N N C P N T V W P G T L T G D Q K
ACCTCAGTTATCACTCACCGGCTTCGAACTAGCATCCAAAGCTAGCCGATCAGTGGACGC 240
49  P Q L S L T G F E L A S K A S R S V D A
TCCATCTCCATGGTCTGGTCTGCTTCTGGGGCCGAACCAGATGCTCCACGGACGCCGCTGG 300
69  P S P W S G R F W G R T R C S T D A A G
AAAATTCACTTGTGAACTGCAGACTGTGGCTCTGGCCAGGTGCGATGCAACGGGGCAGG 360
89  K F T C E T A D C G S G Q V A C N G A G
CGCAGTTCACCAGCAACTTTAGTTGAAATCACAATTGCGGCAAACGGGGGTCAAGATTA 420
109  A V P P A T L V E I T I A A N G G Q D Y
TTATGATGTTAGCCTTGTTGACGGCTTCAACTGCCTATGTCTGTCGCCCCACAAGGTGG 480
129  Y D V S L V D G F N L P M S V A P Q G G
CACGGGCGAGTGCAAGCCCTCGTCTTGCCCTGCCAATGTTAACAAGGTGTGCCCGGCTCC 540
149  T G E C K P S S C P A N V N K V C P A P
ACTTCAAGTGAAAGCGGCTGATGGGAGTGTTCATCAGTTGCAAAGCGCTTGCCTTGCGTT 600
169  L Q V K A A D G S V I S C K S A C L A F
TGGTGATTGGAAGTACTGCTGCACTCCGCCGAATAATACGCCGAGACATGTCCTCCAC 660
189  G D S K Y C C T P P N N T P E T C P P T
AGAGTACTCTGAGATCTTTGAGAAGCAG TGCCTCAAGCTTATAGCTACGCTTATGATGA 720
209  E Y S E I F E K Q C P Q A Y S Y A Y D D
TAAAAACAGCACATTTACCTGCAGTGGTGGACCTGACTACGTCATTACTTTCTGCCATA 780
229  K N S T F T C S G G P D Y V I T F C P - 247
      ↑
agcacgaaatgggattatatatgcagatgatgatatctgtttctttatgtaacaataatg 840
aagaagaataaattccgcggacgttgacacattgctgttgtaagaatttgtaataactaa 900
ttacacgatcaaataaaggaacaaatattatatttaaaaaaaaaa 944

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Fig. 2. Nucleotide and Deduced Amino Acid Sequence of the *Mdt11* cDNA.

The deduced amino acid sequence of *Mdt11* contained the analyzed *N*-terminal sequence (underlined) of the isolated 31-kDa protein. Shaded sequence indicates the putative signal peptide. A putative *N*-glycosylation site (N* S T) is marked by an arrow (N₂₃₀). Forward and reverse primers used in RT-PCR are boxed. This sequence is available from the Genbank database with accession number AF090143.

PCGENE software (IntelliGenetics, Release 6.60) was used for the computer analyses of the nucleotide and amino acid sequences.

Northern and genomic DNA blot analysis. About 5 µg of total RNA from apple fruits and leaves was separated on 1.0% agarose gels containing 15% formaldehyde and blotted onto a Hybond-N membrane

(Amersham). Hybridization and washings were done as described in Sambrook *et al.*²¹⁾ with slight modifications. The open reading frame (ORF) region of *Mdt11* cDNA was labeled with [α -³²P]dCTP. A partial cDNA of 18S rRNA labeled with [α -³²P]dCTP was used as a control probe to measure the relative amount of RNA put into each lane.

Genomic DNA was isolated from young leaves of

Mdt11 (AF090143)	-AKITFTNNCPNTVWPGTLTGDKPQLSLTGFEELASKASRSVDAPSPWS-GRFWGRTRCS	58
CHTL (P50694)	-ATISFKNNCPYMWVPGTLTSDQKPLSTTGFEELASQASFQLDTPVWPN-GRFWARTGCS	58
PsTL1 (AB006009)	-AKFTFTNKCPTVWPGTLTGGGGPQLSLTGFEELASGASTSLTVQAPWS-GRFWGRSHCS	58
PR5K (U48698)	STNFTIENKCDYTVWPGFLMTTAVSLPTNGFSLKKGESRVINVPSPWS-GRWLGRSFCS	59
NP24_LYCES (P12670)	-ATIEVRNNCPYTVWAASTPIG-----GGRRLNRGQTWVINAPRGTKMARIWGRTCGN	52
OS81_SOLCO (P50702)	-ATIEVRNNCPYTVWAASTPIG-----GGRRLNRGQTWVINAPRGTKMARIWGRTCGN	52
Zeamatin (P33679)	-AVFTVVNQCPTTVWAAASVPVG-----GGRQLNRGESWRITAPAGTTAARIWARTGCK	52
VVTL1 (AF003007)	-ATFDILNKCTYTVWAAASPG-----GGRRLDSGQSWTITVNPGTNARIWGRTSCT	51
PRR1_TOBAC (P13046)	-ATFDIVNKCCTYTVWAAASPG-----GGRRLDSGQSWTSINVPNPGTVQARIWGRTNON	51
THAUMATIN (P02883)	-ATFEIVNRCSYTVWAAASKGDA--LDAGGRQLNSGESWTINVEPGTNGGKIWARTDCY	57
	* * * * *	
Mdt11 (AF090143)	TDAAGKFTCETADCGSGQVACNGAGAVPPATLVEITIAANGGQDYDVSLVDGFNLPMSV	118
CHTL (P50694)	TDASGKFVCATADCASGQVMNGNGAIPATLAEFNIAGGGQDFYDVSLVDGFNLPMSV	118
PsTL1 (AB006009)	IDSSGKFVKCTGDCGSGQISONGAGASPPASLVELTLATNGGQDFYDVSLVDGFNLPKL	118
PR5K (U48698)	TSSTGNFSCATGDCGSGGKIEGSDSGARPPTLIDFTLDATDGQDFYDVSVVDGYNLPLVV	119
NP24_LYCES (P12670)	FNAAGRGTCTGDCG-GVLOCTGWGKP-PNTLAEYALDQFSNLDWFIDISLVDGFNIPMTF	110
OS81_SOLCO (P50702)	FNAAGRGSCTGDCG-GVLOCTGWGKP-PNTLAEYALDQFSNLDWFIDISLVDGFNIPMTF	110
Zeamatin (P33679)	FDASGRGSCRTGDCG-GVLOCTGYGRA-PNTLAEYALKQFNNLDFDISLIDGFNVPMSE	110
VVTL1 (AF003007)	FDANGRGKCTGDCN-GLLECGQYGSF-PNTLAEFALNQPNLDYIDISLVDGFNIPMDF	109
PRR1_TOBAC (P13046)	FDGSGRGNCETGDCN-GMLECGQYGKA-PNTLAEFALNQPN-QDFVDISLVDGFNIPMEF	108
THAUMATIN (P02883)	FDDSGSGICKTGDCG-GLLRKRFGRF-PNTLAEFSLNQYG-KDYIDISNIKGFNVPMNF	114
	* * * * *	
Mdt11 (AF090143)	APQ-GGTG-ECKPSSCPANVNKVCAPAPLQVKAADGS--VISCKSACLAFGDSKYCCTPPN	174
CHTL (P50694)	TPQ-GGTG-DCKTASCPANVNAVCPSELQKKGSDGS--VVALCLSAFCVFGTPQYCCCTPPQ	174
PsTL1 (AB006009)	APR-GGSG-DONSTSCAANINTVCPAELSDKGS DGS--VIGCKSACLALNQPYCCTGAY	174
PR5K (U48698)	VPQRLGSGRTCSNVGCVVNLNKTCPSELKVMGSSNKEHPIACMNACQKFLPEFCYGEY	179
NP24_LYCES (P12670)	APTKPSGG-KCHAIHCTANINGECPRALKVPGG-----CNNPCTTFGGQYCCCTQGP	161
OS81_SOLCO (P50702)	APTKPSAG-KCHAIHCTANINGECPRALKVPGG-----CNNPCTTFGGQYCCCTQGP	161
Zeamatin (P33679)	LPDGGG-G-CRGRPCAVDVNARCPAELRQDGV-----CNNACPVFKKDEYCCVGS	160
VVTL1 (AF003007)	S-----GGRGIQCSVDINGQCPSELKAPGG-----CNNPCTVFKTNEYCCTDGP	153
PRR1_TOBAC (P13046)	SPTN---G-GCRNLRCCTAPINEQCPAQLKTQGG-----CNNPCTVIKTNEYCCTNGP	156
THAUMATIN (P02883)	SPTT---R-GGRVRCADIVGQCPAKLKAPEGG-----CNDACTVFQTSYCCCTTG-	162
	* * * * *	
Mdt11 (AF090143)	NTPETCPPTSEYSEIFEKQCPQAYSAYDDKNSTFTCSGG-PDYVITFCP-----	222
CHTL (P50694)	NTPETCPPTNYSEIFHNACPDAYSAYDDKRGFTTNGG-PNYAITFCP-----	222
PsTL1 (AB006009)	GTPDTCPTDFSKVFNQCPQAYSAYDDKSSTFTCFGG-PNYEITFCP-----	222
PR5K (U48698)	GKPAKCPPTLYSTNFKNECPPLAYSYADNENNTFRCSNS-PNYVITFCPNDISSMSQPSK	238
NP24_LYCES (P12670)	-----CGPTELSKFFKKRCPDAYSYPQDDPTSTFTCPGGSTNYRVVFCPNGVADPNFPLE	216
OS81_SOLCO (P50702)	-----CGPTELSKFFKKRCPDAYSYPQDDPTSTFTCPSGSTNYRVVFCPNGVADPNFPLE	216
Zeamatin (P33679)	--ANDCHPTNYSRYFKGQCPDAYSYPKDDATSTFTCPAG-TNYKVVFPCP-----	206
VVTL1 (AF003007)	--G-SGPTTYSKFFKDRCPDAYSYPQDDKTSLFTCPSG-TNYKVVFPCP-----	198
PRR1_TOBAC (P13046)	--G-SGPTDLSRFFKRCPCDAYSYPQDDPTSLFTCPSG-TNYRVVFCP-----	201
THAUMATIN (P02883)	----KGPTSEYSRFFKRLCPDAFSYVLDKPT-TVTCPGS-SNYRVVFCTA-----	207
	* * * * *	
Mdt11 (AF090143)	-----	
CHTL (P50694)	-----	
PsTL1 (AB006009)	-----	
PR5K (U48698)	ETNGGKQKSSWKLK	253
NP24_LYCES (P12670)	MPASTDEVAK----	226
OS81_SOLCO (P50702)	MPASTDEVAK----	226
Zeamatin (P33679)	-----	
VVTL1 (AF003007)	-----	
PRR1_TOBAC (P13046)	-----	
THAUMATIN (P02883)	-----	

Fig. 3. An Alignment of the Amino Acid sequence of PR5/TL Proteins.

The deduced amino acid sequences of the putative mature proteins were aligned by using the Clustal W program and adjusted manually to obtain the best alignment. The conserved amino acid residues are marked with asterisks (*), and gaps with dashes (-). The sixteen cysteine residues involved in disulfide bridges were also conserved in Mdt11 protein (shaded). In the case of PR5K,²⁰ only the sequence of the putative extracellular domain was used for alignment.

apple plants as described in Kim *et al.*²² Isolated genomic DNA (10 µg) was digested completely with *Eco*RI, *Hind*III, or *Eco*RV, separated on a 0.8% agarose gel and blotted onto a Hybond-N membrane (Amersham). Hybridization and washing were done by the same method for the northern blot analysis, using the 3'-untranslated region (UTR) or ORF

region of *Mdt11* cDNA as probes.

Results and Discussion

Identification of a 31 kDa protein abundant in fully-grown apple fruits

Figure 1 shows proteins from apple fruits (A) and

leaves (B) analyzed by 2D-PAGE. Four fruit-specific protein spots appeared abundantly in the flesh of fully-grown fruits at 14 WAP (Fig. 1A). Their molecular weights were about 58, 31, 27, and 19 kDa, estimated from their migrations on the gel.

In order to identify these proteins, we collected them from the 2D-PAGE gels and analyzed their *N*-terminal sequences. The *N*-terminal sequence of the 31-kDa protein was "A K I T F T N N X P N T V X P", which was identical to a partial sequence of an apple food allergen,²³⁾ and similar to the sequences of many PR5/TL proteins involved in the defensive functions of plants.¹⁸⁻²⁰⁾ The *N*-terminal sequences of 58, 27, and 19-kDa proteins could not be analyzed, possibly because of the protein modifications at their *N*-termini.

A cDNA clone (Mdt1l) encoding the 31-kDa protein was isolated

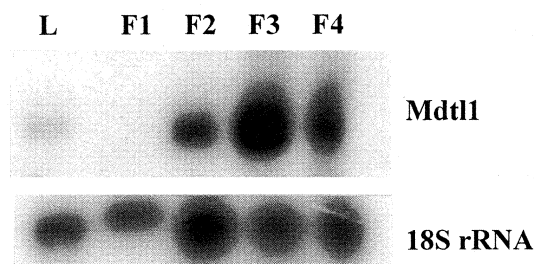
A cDNA fragment of 695 bp was amplified by RT-PCR and used as a probe to clone the gene encoding the 31-kDa protein from a cDNA library of the apple fruit flesh. A cDNA clone of 944 bp was isolated and named *Mdt1l* (*Malus domestica* thaumatin-like protein 1). *Mdt1l* had a 5'-UTR of 37 bp, 3'-UTR of 163 bp, and ORF of 744 bp. The translated ORF contained a putative signal peptide sequence of 25 amino acids and the *N*-terminal amino acid sequence of 31-kDa protein. A putative glycosylation site (N^{*} S T) was found at the position of N₂₃₀ (Fig. 2).

The deduced amino acid sequence of *Mdt1l* shows a significant similarity with many PR5/TL proteins. The highest similarity was with the CHTL protein (70.6%) isolated from ripening cherry fruits¹⁸⁾ and with the PsTL1 protein (65.2%) from the style of the Japanese pear.¹⁹⁾ The sixteen cystein residues involved in disulfide bridges²⁴⁾ were also conserved in the Mdt1l proteins (Fig. 3).

PR5/TL proteins can be categorized into two subgroups. The basic subgroup is localized in the vacuolar compartment, and the slightly acidic subgroup is apoplasmic.²⁵⁾ The *pI* calculated from the deduced amino acid sequence of *Mdt1l* is 5.02, which is consistent with the migration of the protein in the 2D-PAGE gels. Mdt1l protein has a putative signal peptide and lacks the *C*-terminal extension found in the PR5/TL proteins of the basic, vacuolar-targeted subgroup.²⁶⁾ These indicate that Mdt1l is a member of the slightly acidic subgroup and is localized in the extra-cellular space.

The molecular mass of Mdt1l predicted from the deduced amino acid sequence is 23.2 kDa, which is smaller than that estimated from the migration in the 2D-PAGE gels (about 31 kDa). One possibility is that Mdt1l protein could be glycosylated after translation, as reported in the case of the PsTL1 protein from the pistil of Japanese pear.¹⁹⁾ The

(A) Northern blot analysis



(B) Relative expression level

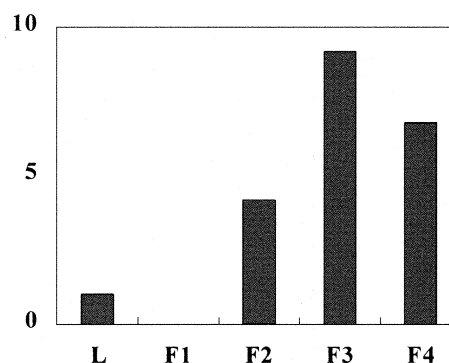


Fig. 4. Expression of *Mdt1l* in Apple Fruits of Various Developmental Stages.

(A) Total RNA (5 μ g/lane) was separated on 1.0% agarose gels containing 15% formaldehyde and blotted onto Hybond-N membrane (Amersham). The membrane was hybridized with the ORF region of *Mdt1l* cDNA labeled with [α -³²P]dCTP. L indicates expanded leaf; F1, young fruit (4 WAP); F2, growing fruit, (8 WAP); F3, fully-grown fruit, (14 WAP); and F4, ripening fruit (20 WAP). Radiolabeled fragment of 18S rRNA was used as a control probe. (B) Graph summary of the signal intensities measured by BAS-1500 phosphorimager (Fujifilm, UK), which shows the relative levels of *Mdt1l* transcript.

putative *N*-glycosylation site found in the deduced sequence of *Mdt1l* supports this assumption. The discrepancy between the predicted molecular mass and the mobility on the PAGE gels was also reported in the case of CHTL from cherry fruit¹⁸⁾ and other proteins.^{16,19)}

Expression of Mdt1l is developmentally regulated

The expression pattern of *Mdt1l* was investigated by northern blot analysis using total RNA isolated from apple leaves and fruits of different developmental stages (Fig. 4). The result indicates that the pattern of *Mdt1l* expression is developmentally regulated. The accumulation of *Mdt1l* transcripts

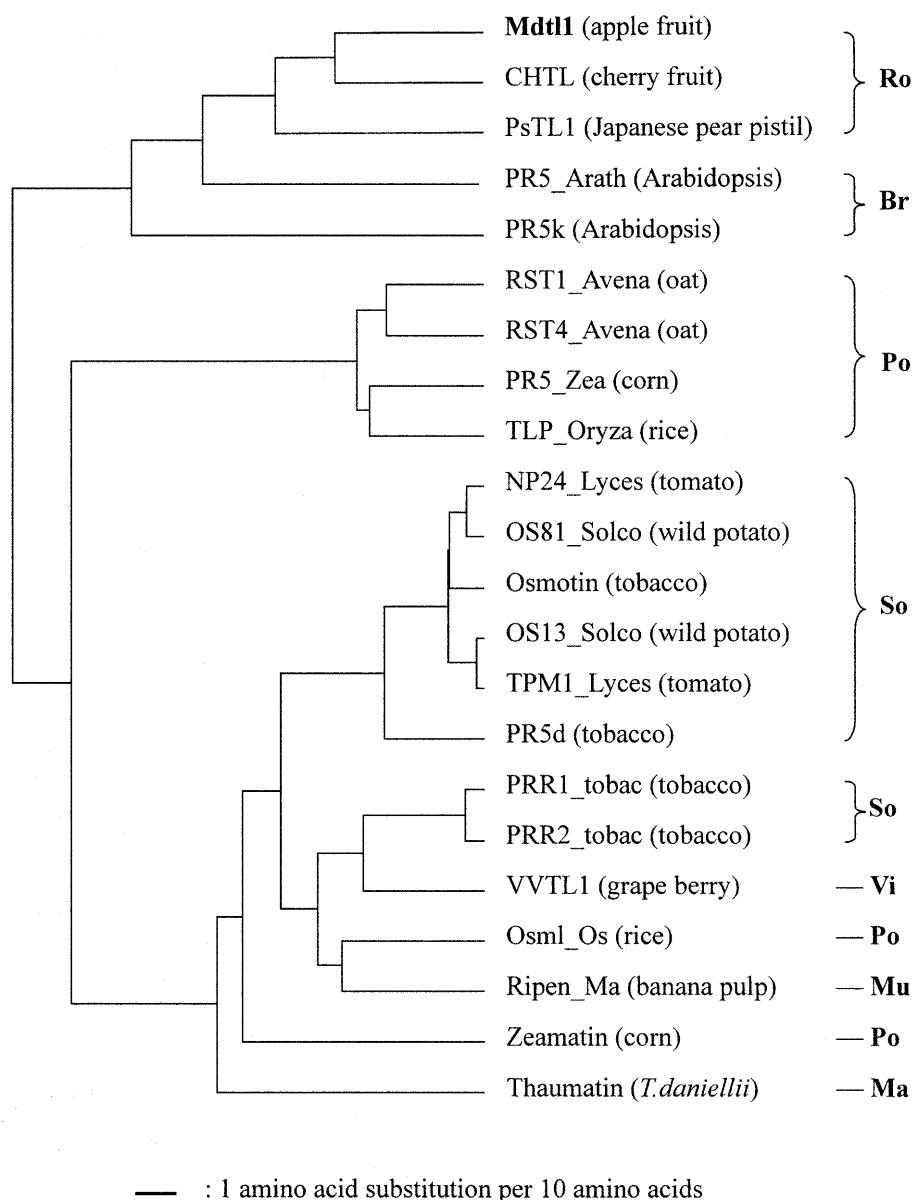


Fig. 5. Phylogenetic Tree of the PR5/TL Proteins.

The tree was constructed by using Wisconsin Package Version 9.0 (Genetics Computer Group). The predicted sequences of mature proteins were used for tree construction. Mdt11 (AF090143), CHTL (P50694), PsTL1 (AB006009), PR5_Arath (AL0223), PR5k (U48698), RST1_Avena (P50695), RST4_Avena (P50698), PR5_Zea (U82201), TLP_Oryza (P31110), NP24_Lyces (P12670), Os81_Solco (P50702), Osmotin (S30157), Os13_Solco (P50136), TPM1_Lyces (Q01591), PR5d (2981950), PRR1_tobac (P13046), PRR2_tobac (P07052), VVTL1 (AF003007), Osml_Os (Y10992), Ripen_Ma (AF001528), Zeamatin (P33679) and Thaumatococcus (P02883). For the PR5K protein,²⁰ only the sequence of the putative extracellular domain was considered. Higher taxa are indicated as follows: **Ro**, Rosaceae; **Br**, Brassicaceae; **Po**, Poaceae; **So**, Solanaceae; **Vi**, Vitaceae; **Mu**, Musaceae; **Ma**, Marantaceae. Scale bar (—) indicates the distance of one amino acid substitution per 10 amino acids.

starts at the beginning of the growth phase with cell expansion (8 WAP) and reaches the peak at the end of the growth phase (14 WAP). The level of *Mdt11* transcripts declines slightly as the fruits begin to ripen (20 WAP). Transcripts of *Mdt11* gene were also detected in expanded leaves, but the expression level was much lower than that of the fully-grown fruits.

PR proteins are typically expressed in low levels in all plant cells but may be induced in higher levels in

response to various physiological stresses.²⁵ However, there are several reports that PR5/TL proteins accumulate in healthy fruits and flowers. VVTL1 in grape berries,²⁷ NP 24 I/II in tomato fruits,²⁸ and CHTL in cherry fruits¹⁸ were isolated and characterized. A ripening-associated cDNA, which is similar in sequence to thaumatococcus, was also isolated from banana pulp.²⁹ The pistils of tomato³⁰ and Japanese pear¹⁹ have been also reported to accumulate PR5/TL proteins in large amounts.

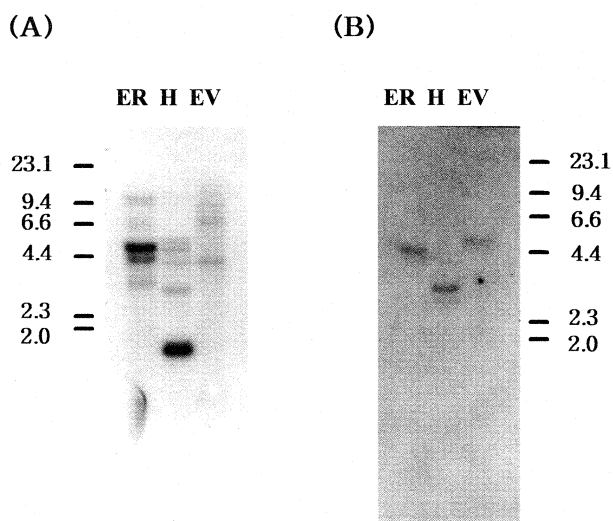


Fig. 6. Southern Blot Analysis of *Mdt11*.

Genomic DNA (10 μ g/lane) was digested with either *Eco*RI (ER), *Hind*III (H) or *Eco*RV (EV), separated on a 0.8% agarose gels and transferred to a Hybond-N membrane (Amersham). The membrane was hybridized with the ORF region (A) or 3'-UTR (B) of *Mdt11* cDNA labeled with [α - ^{32} P]dCTP. Molecular mass standards are shown in kilo-base pairs.

Why PR5/TL proteins are accumulated in healthy organs is yet unclear. It has been known that the expression of PR5/TL protein is related to the accumulation of soluble sugars in the grape berry.²⁷⁾ PR5/TL protein in mature grape berry shows *in vitro* antifungal activities in high sugar concentrations and is thought to be involved in defensive roles in combination with other PR proteins.³¹⁾ However, we failed to detect antifungal activities of *Mdt11*, which is expressed in *E. coli* as described in Hu and Reddy.³²⁾ Recently, it has been known that several PR5/TL proteins bind to or hydrolyze β -1,3-glucans.^{33,34)} Further purification and characterization of intact protein from apple fruits is required to find whether *Mdt11* has antifungal or other enzymatic activities. The accumulation of *Mdt11* coincided with the growth phase of apples, during which the fruit cells undergo rapid expansion and severe changes in turgor pressure.¹⁾ Therefore, we cannot exclude the possibility that *Mdt11* may play some other roles in the development of fruit cells.

Mdt11 is present as single copy in the apple genome

A phylogenetic tree was constructed with the PR5/TL proteins including fruit or flower-specific PR5/TL proteins by the UPGMA method (Fig. 5). *Mdt11* was grouped together with CHTL and PsTL1, which are also acidic PR5/TL proteins with similar molecular masses to the *Mdt11* protein. The expression pattern of CHTL is also similar to that of *Mdt11*,¹⁸⁾ suggesting that *Mdt11* in apple is the counterpart of CHTL in cherry. The presence of PsTL1, a style-specific protein, has not been investigated in

the fruit of Japanese pear.¹⁹⁾ However, it is possible that there exists a PsTL1 counterpart in apple flowers and/or a *Mdt11* counterpart in the fruit of Japanese pear.

The results of the genomic DNA blot analysis also supported the possible presence of other related TL sequences in the apple genome (Fig. 6). Several bands were found in each lane when the genomic DNA was hybridized with the ORF region of *Mdt11* (Fig. 6A). However, a single hybridization band appeared when the digested genomic DNA was hybridized with the 3'-UTR of *Mdt11* (Fig. 6B). These results indicate that the *Mdt11* may be present as a single copy, and there exist some sequences closely related to *Mdt11* in the apple genome.

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