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Ectopic Expression of Apple *MbR7* Gene Induced Enhanced Resistance to Transgenic *Arabidopsis* Plant Against a Virulent Pathogen

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Abstract A disease resistance related gene, *MbR7*, was identified in the wild apple species, *Malus baccata*. The *MbR7* gene has a single open reading frame (ORF) of 3,288 nucleotides potentially encoding a 1,095-amino acid protein. Its deduced amino acid sequence resembles the *N* protein of tobacco and the *NL27* gene of potato and has several motifs characteristic of a TIR-NBS-LRR R gene subclass. Ectopic expression of *MbR7* in *Arabidopsis* enhanced the resistance against a virulent pathogen, *Pseudomonas syringae* pv. *tomato* DC3000. Microarray analysis confirmed the induction of defense-related gene expression in 35S::*MbR7* heterologous *Arabidopsis* plants, indicating that the *MbR7* gene likely activates a downstream resistance pathway without interaction with pathogens. Our results suggest that *MbR7* can be a potential target gene in developing a new disease-resistant apple variety.

Key words: Disease resistance gene, apple, TIR-NBS-LRR (Toll Interleukin 1 Receptor-Nucleotide Binding Site-Leucine Rich Repeat), ectopic expression

Developing an apple cultivar with high-quality fruiting traits and disease resistance has not been an easy task. The *Vf* gene (scab resistance gene) from the wild small-fruited *Malus floribunda* 821 was introgressed into the cultivated apple (*Malus domestica* Borkh.) through an early conventional breeding program [4]. However, this method, first applied in 1914, did not produce any varieties of commercially acceptable fruit trees until 1970 [5].

Recently, researchers have cloned the resistance gene analogs (RGA) such as *Vf* from wild relatives [2, 24]. They were isolated by using heterologous primers in conserved regions for nucleotide-binding site (NBS) motifs [1, 16, 18, 30] found in many resistance genes. However, it is difficult to test the functionality of newly isolated genes in apple directly, because its genus has a long generation time and high chromosome number (2n=34) and it is self-incompatible and highly heterozygous.

In this study, we isolated a TIR (Toll Interleukin 1 Receptor)-NBS-LRR (Leucine Rich Repeat) class of the *R* gene from a wild-type apple, *Malus baccata*, using mixed RGA probes [15]. Since the direct functional test in apple plant is difficult and time-consuming, the function of the isolated *R* gene, putatively named as *MbR7*, was tested in *Arabidopsis*. *MbR7* induced the disease resistance related genes and conferred the disease resistance in *Arabidopsis*.

Materials and Methods

Plant Material, Bacteria Inoculation, and Disease Resistance Scoring

Young healthy leaves of *Malus baccata*, a wild apple species, were obtained from the National Horticultural Research Institute in Suwon, South Korea, and used for the genomic DNA isolation and total RNA preparation.

The transformed *Arabidopsis* seeds of *A. thaliana* ecotype Columbia (Col-0) were planted in moist potting soil and reared for approximately 20 days in a growth chamber at 23°C under constant light before further tests. For large-scale inoculation of the pathogenic bacterium, the surfactant Silwet L-77 (Duchefa) at a concentration of 0.01% (v/v) was added to the suspension (10^7 to 10^8 CFU/ml) of the virulent bacterial strain *Pseudomonas syringae* pv. tomato DC3000, as described in Whalen *et al.* [25]. The leaves of whole plants were dipped in the suspension and on day 5 scored for the presence of necrotic or water-soaked lesions surrounded by chlorosis. To analyze the growth of the pathogen in transformed or non-transformed control plants, five randomly selected leaf discs (0.4 cm diameter) were

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taken, macerated in 10 mM $MgCl_2$, and with the appropriate dilutions plated on fresh agar containing rifampicin and cycloheximide.

Isolation of *MbR7* cDNA Clone and Sequence Analysis

The cDNA library of *M. baccata* was made with a cDNA Synthesis Kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions. This library was screened using a mixed probe of 5 different RGA fragments with the NBS domain isolated in this laboratory [15]. Positive clones were selected and sequenced by terminator sequencing on an automatic DNA sequencer (Bionex Inc. and Core BioSystem Inc., Korea). A sequence homology search was performed with the BLASTX program through NCBI (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was performed using the BCM Search Launcher Clustal W 1.8 Program (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). The putative TIR R gene was named as *MbR7* and its sequence was deposited in the GenBank database under accession number AY363617.

Isolation of the Full-Length *R* Gene by Rapid Amplification of cDNA Ends Analysis (RACE)

Total RNA was isolated from 1 g of apple leaves by the LiCl precipitation method [21]. RACE analysis was performed with the First Choice RLM-RACE kit (Ambion, Austin, TX, U.S.A.) according to the manufacturer's protocol. For the 5' RACE analysis of *MbR7*, we used one anchor-specific primer described in the manufacturer's protocol and two gene-specific primers (outer and inner) -- OLE 2427 (5'-GTCCTGATCCATATAAGCCTGG-3') and OLE1286 (5'-CTTCGTGGGCTGTCATGG-3'). The final PCR products of the 5' RACE reactions were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.), and several independent clones were analyzed.

Isolation of *MbR7* Structural Gene from Genomic DNA

Genomic DNA was isolated from apple leaves using the Nucleon Phytopure plant and fungal DNA extraction kit (Amersham, Piscataway, NJ, U.S.A.) according to the manufacturer's instructions. The structural region of the *MbR4* gene from genomic DNA was amplified by PCR using two primers corresponding to the extreme ends of cDNA sequences: OLE2832 (5'-GTTGATCGAAGGAGCTTGA-CTTGG-3') and OLE2774 (5'-GTCTTCATTATCGAACC-AGTAAAGCG-3'). This PCR product was electrophoresed on agarose gel, purified, and sequenced.

Production of *Arabidopsis* Transgenic Plants Expressing *MbR7*

To express the *MbR7* gene in *Arabidopsis*, the cDNA fragment (3,487 bp containing the complete ORF from +119 to +3606 of AY363617) was amplified by PCR using two primers: OLE1249 (5'-GCTCAGATCTTGTGTGGAAT-3') and OLE1250 (5'-CTGGCGTTACAGATCTTAAT-3'). The amplified PCR product was cloned into a gentamycin and kanamycin resistance-conferring binary vector, pSL5 [14]. A pSL5-*MbR7* recombinant plasmid (*35S::MbR7*) was first introduced by electroporation into the *Agrobacterium tumefaciens* ASE strain, and then transformed into *Arabidopsis* via the floral dip method [3]. The resultant transformed seeds were grown on agar plates supplemented with kanamycin (50 μg/ml); resistant plants were then transferred onto the soil. Plants of T3 generation were used in experiments for pathogen treatment and microarray-based expression profiling analysis.

RT-PCR

The expressions of the *MbR7* gene and pathogenesis-related (PR) gene in kanamycin-resistant T3 plants were confirmed by RT-PCR analysis. To detect the expression of the *MbR7* gene in the transgenic plant, RT-PCR was performed using the gene-specific primers, OLE2286 (5'-GATAAACGAGAGGTCATCAA-3') and OLE2848 (5'-TGACATAATCAAGACAGATGATGC-3'), as described in Lee *et al.* [13]. As a control, a pair of actin-2 primers [a forward primer (5'-GGAATTCCACCATGTTCCCA-3') and a reverse complementary primer (5'-ATTGTCACCCG-ATAC-3')] was used. To detect expression of the *Arabidopsis* thaumatin gene, forward primer OLE1190 (5'-GTGATTC-ATGTACGGCTGCG-3') and reverse primer OLE3315 (5'-ACGCATTACCAATCAATTAGTTGTGC-3') were used. The expression of the endochitinase gene was analyzed using a forward primer, OLE 3317 (5'-CCAGCGAAAGGGTTCT-ACAC-3'), and a reverse primer, OLE3316 (5'-TAGCAA-CTAAGATTTGCTCCAGG-3').

Gene Expression Profiling in the Transgenic Plants by cDNA Microarray

A cDNA microarray containing about 700 cDNA probes of *Arabidopsis* (constructed in this laboratory) was used to analyze the expressions in transgenic plants. The leaf samples from 35S::*MbR7* transgenic *Arabidopsis* (T3) or non-transformed control *Arabidopsis* were homogenized in liquid nitrogen and the total RNA was isolated by the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was labeled by directly incorporating Cy3- or Cy5-conjugated dUTP (Perkin Elmer Life Sciences, MA, U.S.A.) during reverse transcription. The overall procedure of hybridization and washing was performed according to the protocol described at http://cmgm.stanford.edu/pbrown. After washing, the slides were immediately scanned in ArrayWoRx (Applied Precision, Issaquah, WA, U.S.A.). Intensity values were obtained using ImaGene image analysis software (BioDiscovery, Los Angeles, CA, U.S.A.) and were analyzed by the GeneSight software package and various softwares described in Kim *et al.* [12] and Oh *et al.* [19]. Analyses were performed

with mean signal intensity values assigned for each spot. The ratio of the mean hybridization intensity for each element was normalized by dividing them with the mean of the selected subset (β-tubulin and actin-2).

RESULTS

Isolation of *MbR7*, a NBS-LRR-Type *R* Gene, from Wild Apple Species *Malus baccata*

To isolate the NBS-LRR class of the *R* gene from *Malus baccata*, a cDNA library (initial PFU 1.4×10^6) was constructed and screened with a mixed probe of five RGAs with NBS domain (Accession Nos. AF516645, AF516646, AF516647, AF516648, and AF516650) isolated in this laboratory [15]. The nucleotide sequence of the selected cDNA clone was determined and analyzed with BLASTX algorithms in GenBank. This putative R gene, named *MbR7*, contained the conserved domains of the TIR-NBS type of *R* gene and shared high sequence similarity with the TMV resistance *N* gene of tobacco and the *NL27* gene of potato.

Structure of *MbR7* Gene

The isolated *MbR7* cDNA fragment, 3,553-bp-long in size, seemed to be slightly smaller than the size expected by the Northern analysis, 3.6 kb (data not shown). To make sure if the 5' end was correct, 5'-RACE analysis was carried out. The RACE analysis revealed the more complete cDNA clone of 3,606 nt with a size agreed to by the Northern blot analysis. *MbR7* consists of a 3,288 nt putative open reading frame (ORF), potentially encoding a 1,095-amino acid protein, a 119 nt 5' UTR, and a 199 nt 3' UTR. The deduced amino acid sequence showed strong homology with potato *NL27* (40% identity, 62% similarity) and tobacco *N* (40% identity, 57% similarity). The predicted protein had a putative NBS domain (P-loop, kinase 2, and kinase 3a sequences), a domain with unknown function including sequences similar to GLPL, CFLY, and MHD sequence motifs, and a LRR domain (Fig. 2B). In addition, it has an approx. 300-residue region that shares homology with the Apaf-1 and CED-4 activators of apoptosis in animal cells [32]. The putative LRR domain of the *MbR7* gene had 8 imperfect repeats of the cytoplasmic LRR consensus sequence. The LRR region of the *MbR7* gene had all the characteristic sequences of the LRR region involved in the recognition of the avirulence product of the pathogen [20], axxLxxLxLxxC/Nxxa, where "x" designates any amino acid and "a" designates the positions of aliphatic amino acids, followed by a region of varying length. However, the *MbR7* gene had a slightly different motif of NxL(T/S)G with a glycine and N-glycosylation sequence NX(S/T) within the consensus sequence that is consistent with that of the plant extracellular LRR family.

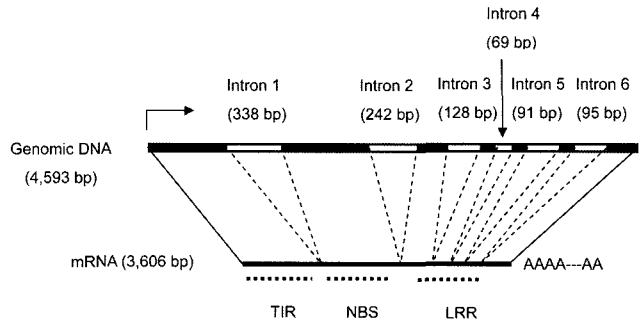

Fig. 1. Schematic representation of the structure of the MbR7 protein.
Exon regions and intron regions from its genomic flanking sequences are indicated by the hatched box. The conserved domains (TIR, NBS, LRR) are indicated by the dashed lines.

Structure of *MbR7* Genomic Gene

From PCR amplification using a set of primers corresponding to the each end of the full-length cDNA, the genomic fragment of *MbR7* was isolated and its sequence was analyzed. As shown in Fig. 1, the *MbR7* gene consisted of seven exons (545, 1093, 279, 740, 37, 237, and 357-bp, respectively) divided by six introns (338, 242, 128, 69, 91, and 95 bp, respectively).

Expression of the *MbR7* Gene in *Arabidopsis* Confers Resistance to *Pseudomonas syringae* pv *tomato*

To verify if *MbR7* functions as a *R* gene, the *MbR7* ORF was cloned in an expression vector and transformed into *Arabidopsis* seeds, and transgenic plants were produced. *MbR7*-specific transcripts were detected by RT-PCR using a *MbR7* primer pair, OLE2286 and OLE2848, in the *MbR7*-transgenic line (*35S::MbR7*#1), but not in either the non-transformed control plants nor in the vector-only (*35S::pSL5*) transgenic plants (Figs. 2A and 2B). There was no detectable difference in growth and the shape of the

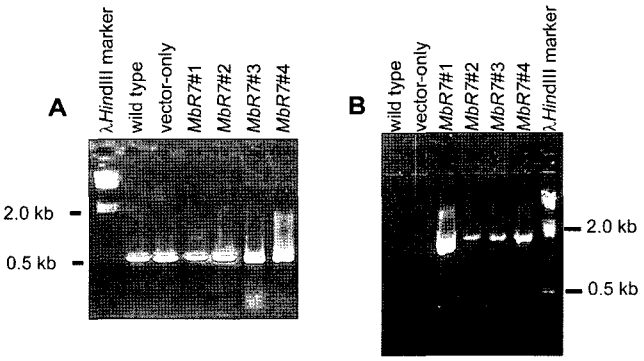

Fig. 2. Overexpression of *MbR7* gene in transgenic *Arabidopsis* lines (T3). Expression of actin as a positive control (**A**) and the *MbR7* gene in transgenic plants (**B**) were confirmed by RT-PCR analysis.
RT-PCR was performed using total RNA from leaves of *35S::MbR7* transgenic *Arabidopsis* plants and gene-specific primers.

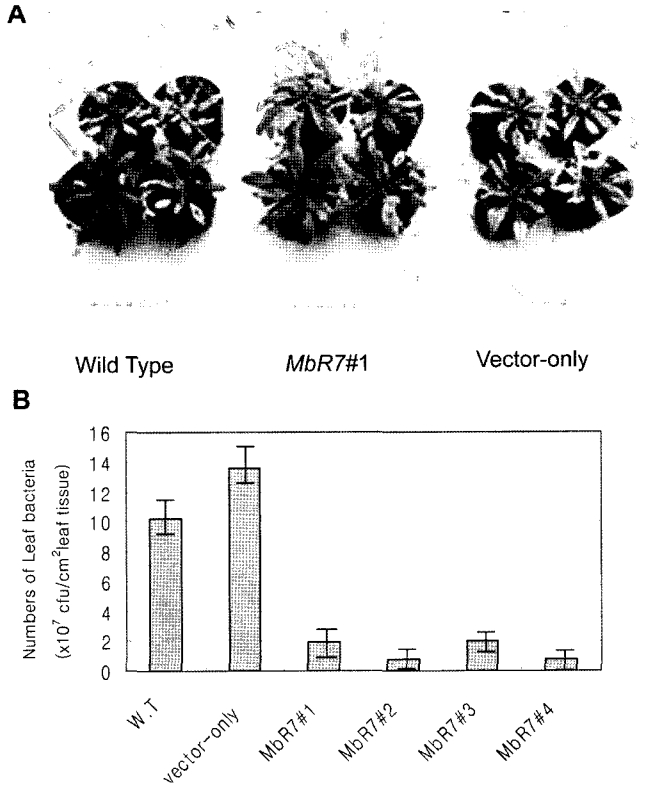

Fig. 3. Analysis of disease resistance against the *P. syringae* pv. *tomato* bacteria in wild-type and vector-only as control plants, and in *35S::MbR7* transgenic plants.
A. Resistant phenotype of *MbR7* transgenic *Arabidopsis* and susceptible phenotype of wild-type and vector-only transgenic plants after inoculation with *P. s. tomato* DC3000 strain. **B.** The growth of *P. s. tomato* DC3000 strains in wild-type (Col-0) and vector-only (35S::pSL5), *35S::MbR7* transgenic plants was compared by determining the number of bacteria. Values are an average of three different experiments. A significant difference was observed between the control and transgenic plants (*t*-test, $P>0.001$). Error bars indicate ±SE.

transgenic plants compared with the wild-type. The *MbR7*-transgenic, vector-only transgenic, and wild-type plants had been treated with a virulent bacteria, *P. syringae* pv. *tomato* DC3000, and the disease symptoms and the growth of the bacteria were monitored. The wild-type or vector-only transgenic plants induced strong disease symptoms, producing gray-brown lesions with chlorosis that spread out from those lesions (Fig. 3A). Although mild chlorosis or necrosis occasionally developed on leaves of the *35S::MbR7*#1 transgenic plant, the lesions with chlorosis were drastically less and much smaller than those in the control plants.

The reduction of the disease symptoms was most likely due to the inhibition of the growth of the pathogen. The number of the bacteria in the leaves of the *35S::MbR7*#1 transgenic plants was 5- to 17-fold less than in the control plants ($P<0.001$) (Fig. 3B). Therefore, overexpression of *MbR7* effectively suppressed the growth of bacterial pathogens in *Arabidopsis*, thereby enhancing resistance.

Altered Expression of Plant Defense-Related Genes in Transgenic Plants

To evaluate expression changes of defense-associated and regulatory genes in this 35S::*MbR7* transgenic plant, microarray-based expression profiling analysis was carried out. Correlation coefficients for the expression values from the *35S::MbR7* line *vs.* the wild-type and for those from the *35S::pSL5* line *vs.* the wild-type were 0.81 and 0.86, respectively, suggesting that not only *MbR7* transgenic plants but also vector-alone plants had altered the overall expression patterns. However, the overall expression level in the *35S::MbR7* plants was much higher than in the vector-alone *35S::pSL5* line (data not shown).

For the detailed analysis of the expression pattern in defense-related genes, another set of microarray of about 170 defense-related genes was analyzed further, including plant defense-related proteins such as PR5 (putative thaumatin protein), endochitinase, glycine-rich cell wall protein, glutathione S-transferase (GST30), polygalacturonase, pectinase, and beta-amylase enzyme. Out of 170 genes, 21, including those for thaumatin protein and endochitinase, were induced far more than two-fold in *35S::MbR7*#1, whereas 5 genes in *35S::pSL5* were more or less induced about two-fold (Fig. 4 and Table 1). Genes that were induced more than two-fold in the transgenic plants or the vector-only transgenic plants are listed in Table 2 and Table 3, respectively. The induction of several PR genes, characteristically known as thaumatin protein and endochitinase, were confirmed by RT-PCR in transgenic *Arabidopsis* plants that overexpressed *MbR7* (Fig. 5). Our observations strongly suggest that even under pathogen-free conditions, the overexpressed *MbR7* gene upregulates expression of a number of PR genes that are normally upregulated in response to a pathogen attack.

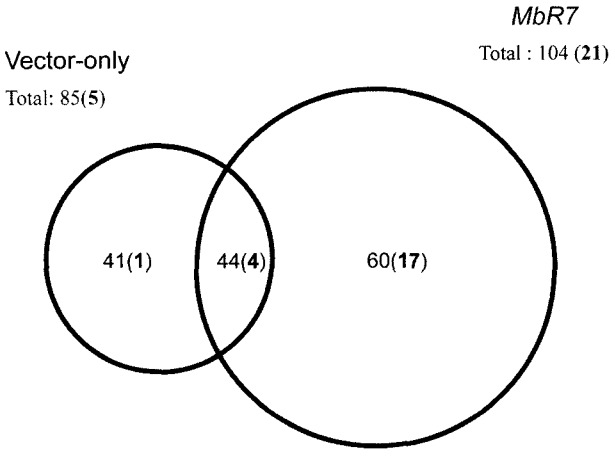

Fig. 4. Expression profile analysis of the overexpressed *MbR7* inducible or regulated gene in 35S::*MbR7* transgenic plants.
The numbers in the Venn diagram indicates the number of induced genes, a minimum 2-fold ratio in each transgenic line. The bold numbers in parentheses indicate defense-associated and regulatory genes.

Table 1. Expression of the selected defense-related genes in the *MbR7* or vector-only transgenic plants.

Clone name	*MbR7*	Ratio*	Vector-only	Ratio*
S33		6.04		1.18
MEK kinase (MAP3Ka)				
LIU45		6.01		-0.17
putative thaumatin protein				
G99		5.75		1.54
polygalacturonase 5				
S35		6.33		1.82
fatty acid desaturase in plastids				
G23		6.55		0.46
putative cinnamoyl-CoA reductase				
G112		7.00		1.55
putative GDSL-motif lipase/hydrolase				
G72		6.14		1.15
putative endochitinase				
LIU61		7.81		1.12
DFR gene for dihydroflavonol 4- reductase				
S17		0.78		1.94
MEK kinase (MAP3Ka)				
ACTIN-2		0		0
actin-2				

*Transgenic/non-transgenic.

DISCUSSION

In this study, we have identified and characterized a novel TIR-NBS-LRR class of *R* gene, *MbR7*, from the wild apple species *Malus baccata*. The complete transcript of *MbR7* was 3,606-bp long in size; the genomic gene consisted of six introns and seven exons. Putative proteins encoded by the *MbR7* gene were most similar to the TMV virus resistance *N* gene of tobacco [26], *NL25* or *NL27* gene of potato [9], and *SNC1* gene of *Arabidopsis* [31], all of which have the TIR-NBS-LRR type of structures. We also confirmed the elevated expression of several defense-related gene expressions and the enhancement of resistance to pathogens in transgenic plants, where *MbR7* was over-expressed.

Recent agricultural trials have proven that the genetic resistance approach is an efficient way to increase crop yields. The ectopic expression of *MbR7* in *Arabidopsis thaliana* resulted in significantly reduced disease symptoms against the plants infected by virulent *P. s. tomato* DC3000 strains (Fig. 3). Moreover, the transgenic plants maintained healthy normal growth even when not exposed to the bacteria. Previously, when several *R* genes have been overexpressed in other plant species via *Agrobacterium*-mediated transformation, those heterologous plants have often either developed necrotic symptoms or altered developmental traits even in the absence of a pathogen attack, possibly due to the interruption of finely tuned signaling pathways in their native species by the transgenic R proteins [23].

Table 2. Genes induced in *MbR7* transgenic plants.

GeneBank no.	Ratio*	Identity	Clone name
AAG51965	6.04	MEK kinase (MAP3Ka)	S33
AT4g36010	6.01	Putative thaumatin protein	LIU45
D17578	6.33	Fatty acid desaturase in plastids	S35
AJ003135	5.75	Polygalacturonase 5	G99
AJ003135	2.89	Polygalacturonase (PGA3)	G98
AF062915	7.69	Putative transcription factor (MYB90)	LIU87
At2g19050	7.00	Putative GDSL-motif lipase/hydrolase	G112
At2g33590	6.55	Putative cinnamoyl-CoA reductase	G23
At4g22880	6.52	Putative leucoanthocyanidin dioxygenase (LDOX)	G9
AF043528	6.45	20S proteasome subunit PAG1 (PAG1)	L58-1
D63460	6.29	Alcohol dehydrogenase	L48-1
AF197940	4.71	SAM:phospho-ethanolamine *N*-methyltransferase (NMT1)	LIU123
AB033294	7.81	DFR gene for dihydroflavonol 4-reductase	LIU61
At1g72340	6.14	Putative endochitinase	G72
AY062447	5.83	Polygalacturonase; pectinase	PIU4
U43713	5.71	Strictosidine synthase (SS) gene	PIU3
AF288191	2.58	Glutathione S-transferase (GST30)	LIU58
AY056244	7.37	Putative glucosyltransferase	G69
AJ250341	7.12	Glucose-6-phosphate/phosphate-translocator precursor	G54
AJ223189	5.12	Beta-amylase enzyme (ct-bmy gene)	S46
AY042874	2.76	PGA2	G106

Specific *R* gene can confer species-specific or broad-spectrum resistance. Considering the overall upregulation of disease-related genes, it is likely that *MbR7* might act broad spectrum. Elevation of disease resistance in heterologous plants by *MbR7* also suggests that the *MbR7* gene likely activates a downstream resistance pathway without interaction with pathogens. The broad-range induction of the native defense-related genes in *Arabidopsis* by *MbR7* also supports the notion that *MbR7* might act as a broad-spectrum disease resistance gene.

The number of clones with more than two-fold induction ratios in 35S::*MbR7*-transgenic plants was much larger than those for vector-only transgenic plants. It is especially noteworthy that expression was three times greater than those of vector-only plants, clearly indicating that *MbR7* plays a role in plant defense. Although most of these genes were activated when the plant was attacked by herbivores and pathogens, even without pathogen infection, our 35S::*MbR7*-transgenic plants induced several significant defense genes through microarray analysis (Tables 1, 2, and 3), such as those involved in gene-encoding signal transduction proteins, and downstream defense genes. The latter includes those encoding pathogenesis-related (PR) protein and enzymes involved in the generation of phytoalexins, lignifications, endochitinase [6, 8], glycine-rich cell wall protein [29], polygalacturonase (pectinase) [6], and beta-amylase enzyme [7]. Proteins related to fatty acid signaling and metabolism, such as fatty acid desaturase [11] and putative GDSL-motif lipase/hydrolase [10], were also expressed. Finally, as shown by our RT-PCR analysis of the thaumatin gene and endochitinase gene (encoding PR protein), the 35S::*MbR7*-transgenic plants showed high expression levels of these genes compared with vector-only transgenic plant.

The 35S::*MbR7*-transgenic *Arabidopsis* may be a model system for analyzing resistance against pathogens without any discernable inhibition in plant growth. Although we have not yet determined whether and how the 35S::*MbR7* gene is activated in apple species, the functional test of the *Bs2* gene from pepper and the *Pto* gene from tomato were carried out in heterologous systems and shown to be good examples [22, 23, 28, 29]. The *MbR7* gene can be a good

Table 3. Genes induced in vector-only transgenic plants.

GeneBank no.	Ratio	Identity	Clone name
AY056244	2.98	Putative glucosyltransferase	G69
AF159801	2.68	Lipid transfer protein 4	LIU19(L)
AJ250341	2.45	Glucose-6-phosphate/phosphate-translocator precursor	G54
AJ223189	2.33	PGA2	G106
AY042874	2.00	Beta-amylase enzyme (ct-bmy gene)	S46

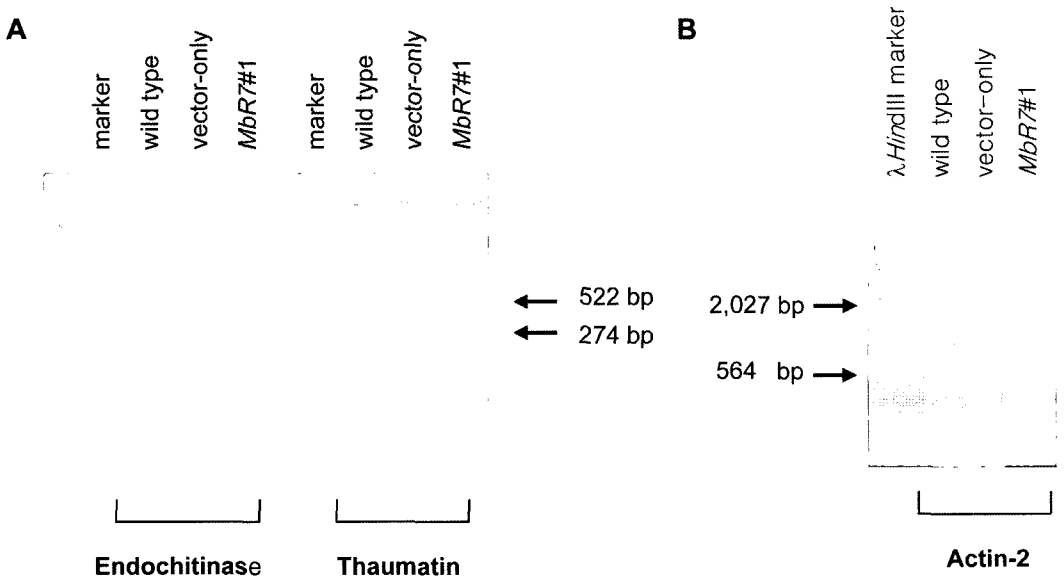

Fig. 5. RT-PCR analysis of (**A**) endochitinase, thaumatin, and (**B**) actin-2 transcripts in 35S::*MbR7* transgenic *Arabidopsis*.
Leaves of vector-only and *MbR7*#1 were harvested from kanamycin-resistant T3 plants. The sizes of endochitinase transcripts and the thaumatin transcripts are 522 bp and 274 bp, respectively.

candidate to confer disease resistance to apple cultivars and improve the yield.

Acknowledgments

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