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Isolation of Defense-Related Genes from *Nicotiana glutinosa* Infected by Tobacco Mosaic Virus Using a Modified Differential Screening

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Many of plant defense responses are consequence of transcriptional activation of related genes. We have developed a modified differential screening procedure to isolate tobacco genes that are involved in the defense responses against TMV infection. A cDNA library was constructed from *Nicotiana glutinosa* leaves infected by TMV under temperature shift conditions. Each of plasmid DNA in the library was hybridized on a set of slot blots to a pool of cDNA probes prepared from either TMV-infected or mock-treated tobacco leaves. Among 900 plasmid DNAs, 81 clones exhibiting significantly enhanced or reduced level of hybridization to either probe were selected for nucleotide sequencing. The clones were listed into 61 genes considering redundancy between the sequences. The genes were identified to be defense-related genes including PR-genes and genes involved in primary or secondary metabolisms. This result supports the implication that plant defense process entails a major shift in total cellular metabolisms rather than activation of a limited number of defense-related genes. Expression patterns of a number of selected genes were examined in northern blot analyses. It is notable that the clone 630 of unknown function exhibits expression pattern similar to those of previously known PR-genes. Experiments to elucidate the roles in defense mechanism of a couple of genes newly identified in this study are in progress.

Keywords **:** defense-related genes, differential screening, *Nicotiana tabacum*, plant defense, tobacco mosaic virus.

Plants defend themselves against invading pathogens by exerting diverse cellular responses. The defense responses include rapid death of plant cells at the site of infection, so called hypersensitive response (HR), to limit supply of further nutrients for the pathogen growth (Dangl et al., 1996). The HR is accompanied by a large set of defense responses, including generation of reactive oxygen species (Levine et al., 1994; Mehdy, 1994), cell wall fortification by lignin accumulation (Whetten and Sederoff, 1995), and biosynthesis of antibiotics termed phytoalexins (Darvill and Albersheim, 1984; Dixon, 1986). In addition to the local responses, systemic acquired resistance (SAR) develops in uninfected parts of the plant, which provide pre-formed resistance against further infection with a broad spectrum of pathogens (Ryals et al., 1996).

The plant defense responses are consequence of transcriptional activation of defense-related genes (Lamb et al., 1989). Accumulation of the gene transcripts generally commences within minutes to hours for genes activated locally around infection sites, and several hours or days later for genes responding systemically. Through intensive studies on the mechanisms, lots of genes and proteins involved in this process have been identified to establish some important cellular mechanisms leading the defense responses (Hammond-Kosack and Jones, 1996).

In many cases of plant's local and systemic responses to pathogen, a large group of pathogenesis-related (PR) proteins are synthesized in high amounts to display a broad spectrum of antimicrobial activity (Bowles, 1990). In addition to the genes directly related to the defense responses such as PR-genes, transcription of the genes encoding enzymes involved in secon-dary metabolic pathways are stimulated. Most intensively studied secondary metabolisms in this regard are terpenoid and phenylpropanoid pathways for producing phytoalexins and phenolics (Dixon and Lamb, 1990). Moreover, since the secondary metabolism cannot occur without related primary metabolism where large carbon fluxes are supplied, genes involved in primary metabolisms are also expressed. For instance, expression of the genes encoding enzymes mediating the isoprenoid pathway is strongly enhanced by pathogen infection (Choi et al., 1992 and 1994). Similarly, genes for the activated methyl cycle have elevated transcriptional activity, possibly to provide the activated methyl groups to be used in ethylene production and numerous methylation steps for secondary product formation (Kawalleck et al., 1992).

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Thus, complexity of the plant defense mechanisms is becoming apparent, since pathogen defense entails a major shift in metabolic activity rather than altered expression of a few classes of defense-related genes (Somssich and Hahlbrock, 1998). Therefore, identification of a complete set of genes involved in the defense process is an essential step toward understanding of whole scheme of plant defense mechanisms. In this regard, as recently revealed by genetic studies with *Arabidopsis*, many more functionally unidentified plant genes must exist whose products are also required for mounting an effective defense responses (Glazebrook et al., 1996; Rogers and Ausubel, 1997).

Subtractive hybridization, differential screening and differential-display analysis have been developed to isolate differentially expressed genes. Differential screening procedure is a technique to identify induced or repressed genes by screening of a cDNA library using each pool of cDNAs generated from different conditions as a probe. Differential display is a simple and highly sensitive to detect mRNAs of low abundance, whereas differential screening is able to isolate full length of cDNAs (Liang and Pardee, 1992).

To contribute to the goal of most plant-pathogen interaction studies, we have performed differential screening experiments with some modification to isolate genes differentially expressed during plant defense responses. Applying the temperature sensitive nature of tobacco disease resistance, genes induced or repressed upon infection with tobacco mosaic virus (TMV) were isolated. The isolated genes were classified into previously identified defense-related genes, genes encoding primary or secondary metabolic enzymes of known function, and novel genes newly identified in the present study. Expression patterns of some known PR-genes and two new genes, the clones 215 and 630, were examined using RNA blot analyses.

Materials and Methods

Plant materials and treatments. TMV-resistant wild species of tobacco *N. glutinosa*, TMV-susceptible (*N. tabacum* cv. SR1) and resistant (*N. tabacum* cv. Xanthi nc) cultivars were grown in the culture room under conditions described (Yi et al., 1999). The tobacco strain of TMV used in this study was obtained from Dr. E. K. Park at the Korea Research Institute of Ginseng and Tobacco, Korea. For the induction of acute HR by temperature shift (Weststejin, 1981, Kang et al., 1998), ten week-old tobacco plants were inoculated by sap prepared from TMV infected tobacco leaves and placed at 32°C for 48 h for systemic spreading of viruses. The temperature was then shifted to 25°C to induce the systemic hypersensitive response (HR). For mock inoculation as a control, tobacco leaves were mechanically wounded using carborundum and the plants were incubated as described above for the TMV treatment. At the indicated time after the treatments, the leaf tissues were harvested and immediately frozen in liquid nitrogen to store at -80°C

Construction of cDNA library. A cDNA library was constructed with mRNAs isolated from the TMV-inoculated *N. glutinosa* leaves exhibiting apparent HR. Total RNA was isolated using a method previously described (Parish and Kirby, 1966). PolyA$^+$ mRNAs were isolated from the total RNA using oligo-dT cellulose (Boehringer Mannheim, Germany). The cDNA library was constructed using a λZAP cDNA synthesis kit (Stratagene, USA) following manufacturer's instruction.

Modified differential screening. The cDNA library was excised *in vivo* (Stratagene manual), and two or three μg of recombinant plasmid DNA isolated from each clone was loaded on a set of duplicated slot blots. Each of the blots was hybridized to a pool of ^{32}P-labeled cDNA probes. One pool of cDNA probes was prepared from TMV-infected tobacco leaves, and the other was from mock-treated healthy leaves. Hybridization and washing was conducted in 5X SSC containing 50% formamide at 42°C. After exposure to X-ray film, differences in hybridized signal intensities on the two blots were monitored. cDNA clones having enhanced or reduced levels of signal intensity were selected for further experiments.

Nucleotide sequencing and sequence analyses. Partial nucleotide sequences of the clones obtained from the modified differential screening were determined using an automated DNA sequencer (ABI). Analyses of the nucleotide sequences and their deduced amino acid sequences were performed using DNAsis and Prosis programs (Hitachi, Japan). Homology search was performed using BLAST programs to check GenBank, EMBL, and SwissProt databases.

Northern blot analysis. Twenty mg of total RNA from each sample was fractionated on a formaldehyde-containing agarose gel and transferred onto Nytran membrane (Amersham, USA). Loading of equal amount of RNA was confirmed by probing of ^{32}P-labeled ribosomal DNA. The RNA blots were hybridized to ^{32}P-labeled cDNA probes obtained from each clone at 42°C in 5X SSC containing 50% formamide. The blots were washed under the same condition except that 2X SSC was used.

Results

Modified differential screening. Leaves of ten-week-old tobacco plants were inoculated with TMV or mock-treated and the plants were placed under temperature shift conditions (Weststejin, 1981; Kang et al., 1998). Typical HR lesions appeared about 48 h after the TMV inoculation to the resistant tobacco cultivar *N. tabacum* cv Xanthi nc, while mock-treated control did not show any response (Fig. 1). The susceptible cultivar *N. tabacum* cv SR1 exhibited typical symptom of tobacco mosaic disease upon the TMV infection.

From the cDNA library constructed with TMV-infected tobacco leaves on which HR lesions were apparent, 900 single colonies were selected for the differential screening experiment. Recombinant plasmid isolated from each of the

clones was loaded on a set of slot blots and hybridized to cDNA probes prepared from either TMV-infected or mock-treated tobacco leaves. Two PR genes used as controls, SAR8.2 and PR-1, exhibited higher hybridization signal intensities on the blot probed with TMV-infected tobacco cDNA pools (Fig. 2). From the screening experiment, 81 clones exhibiting different levels of signal intensity on either blot were selected. Partial nucleotide sequence (400-500 bp) of the cDNA contained in each of selected clones was determined.

Homology search for the selected clones. The partial nucleotide sequences were deduced to amino acid sequences and applied to BLASTX analyses to examine their homology with genes previously reported to the databases. Considering redundancy between the sequences, the 81 selected clones were listed into 61 genes (Table 1). Of the 81 clones selected from the modified differential screening, 66 clones (46 genes) showed strong homology with previously identified genes of known function in their amino acid sequences (Table 1 and 2). Among them, 25 clones (12 genes) were classified to known defense-related genes, and 41 clones (34 genes) were to genes encoding proteins involved in primary or secondary metabolisms. Other 15 clones (15 genes) matched to reported genes with low degree of homology.

The 12 defense-related genes include PR-genes encoding PR-1a, PR-3 (acidic chitinase), PR-4b, PR-5 (taumatin or osmotin) and SAR8.2 (Table 2). Genes encoding glutathione-*S*-transferase, glycine-rich protein, gamma thionin and proteinase inhibitor were also classified to the group of defense-related genes. Genes encoding acidic chitinase (PR-3), glutathione-*S*-transferase and glycine-rich protein showed high degree of redundancy. Among the genes encoding proteins of basic metabolisms, RUBISCO small subunit showed very high degree of redundancy. Genes encoding proteins homologous to Cab binding proteins, a ribosomal protein and a metallothionein were also highly expressed in the TMV-infected tobacco leaves (Table 2).

Expression patterns of the identified genes. Some of the genes identified in the differential screening experiment were selected for northern blot analysis to examine their expression patterns during the defense response. PR-genes such as PR-1, PR-5 and SAR8.2 were strongly expressed 48 h after the TMV infection (Fig. 3). Acidic chitinase gene known as PR-3 was also strongly induced throughout the time course of the defense response. Transcripts of this gene were increased in the mock- treated control, suggesting that this gene might be also induced by wounding but not as quickly as by TMV infection. Expression pattern of the phosphoglucomutase (PGM) gene was similar to that of the chitinase gene. The PGM gene was slightly induced by wounding, but strongly expressed 48 h after the TMV infection. Expression of a proteinase inhibitor gene (clone 676) was induced either by mock- or TMV-treatment, but with higher degree in the mock-treated leaves after 24 h after the treatment.

The clone 215 showed high homology with some of previously identified proteinase inhibitor genes. However, expression pattern of this gene was quite different from other known proteinase inhibitor genes as shown with the clone 676. The clone 215 was not significantly induced by wounding as observed in the mock-treated tobacco leaves, but transiently induced 6 to 12 h after the TMV infection when HR is not apparent on the treated leaves (Fig. 3). In addition, the clone 630 was induced 48 h after the TMV infection, which was similar to the expression pattern of typical PR-genes.

Some of the clones showed reduced levels of transcripts upon the TMV infection (Table 2). Transcripts of the clones 156 (putative plastidic aldolase), 512 (gamma thionin) and 60 (plastocyanin) genes were gradually reduced, exhibiting the lowest level at 48 h after the TMV infection (Fig. 4).

Discussion

The TMV-resistant variety of tobacco *N. tabacum* cv. Xanthi nc exerts severe HR response following inoculation with TMV (Fig. 1). The *N*-gene carrying tobacco plants allow TMV to spread systemically without developing HR at 32°C, but exhibit apparent HR lesions when the temperature is shifted to lower than 28°C (Weststejin, 1981). Using the temperature sensitive nature of disease resistance in tobacco, we have observed remarkable changes in expression of diverse genes during the defense responses. Through the homology search for functional analysis of the genes identified in the modified differential screening experiments, it has been observed that expression of various genes involved in diverse primary and secondary metabolisms are altered during the defense process (Fig. 3). Our data confirm previous reports, suggesting that pathogen defense entails a major shift in metabolic activity rather than altered expression of a few unique defense-related genes (Somssich and Hahlbrock, 1998).

As expected, transcription of genes directly involved in defense responses was induced by the TMV infection. PR-genes, including PR-1, PR-3 (acidic chitinase), PR-4, PR-5 (taumatin or osmotin) and SAR8.2, were significantly activated upon the TMV infection at the same time point that HR was apparent on the infected leaves (Fig. 3). The PR-protein was initially termed to describe extracellular proteins that accumulated in response to TMV infection of susceptible tobacco plants, but has been broaden its definition to include intra- and extracellular proteins that accumulate in intact plant tissue or cultured cells after pathogen attack or elicitor treatment (Bowles, 1990). Some of tobacco PR

Fig. 1. Typical symptoms of tobacco plants against TMV infection. The tobacco plants were sap-inoculated and placed under 25°C growth chamber. Left, mock-treated healthy TMV-resistant tobacco *Nicotiana tabacum* cv. Xanthi nc; Middle, same variety of tobacco infected with TMV; Right, TMV-susceptible cultivar *N tabacum* cv. SR1 infected with TMV.

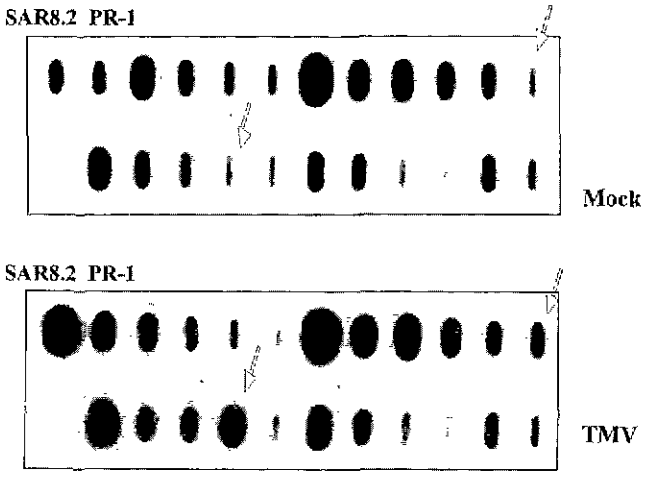

Fig. 2. A typical reverse northern blot hybridization for isolation of differentially expressed genes. Two identical blots were separately hybridized with ^{32}P-labeled cDNA probes prepared from RNA isolated 48 h after either TMV-infected or mock-treated tobacco leaf tissues. Arrows indicate the clones exhibiting enhanced level of mRNA in tobacco leaf tissues developing HR.

proteins are known to be β-1,3-glucanases (Kauffmann et al., 1987) and chitinases (Legrand et al., 1987) that can alter the fungal cell wall architecture to arrest or severely impair fungal growth. SAR8.2 is a basic protein family that is induced during onset of SAR with a expression pattern distinct from other SAR genes (Ward et al., 1991; Alexander et al., 1992). Transgenic plants overexpressing some of PR proteins exhibit increased resistance to fungal pathogens (Broglie et al., 1991; Lui et al., 1994). It has been reported that when two or more PR proteins are constitutively coexpressed, a synergistic increase in the resistance can be obtained (Zhu et al., 1994), suggesting that coordinated activity of several PR genes is necessary for full resistance. In this regard, it is notable that the clone 630 identified in the present study shows an expression pattern similar to that of other known PR-genes (Fig. 3). This gene showed no homology with any other known gene in amino acid sequence, thus a novel gene. Further characterization of the clone 630 to elucidate the role of the gene as a PR-gene is in progress.

The clone 358 encoding a putative acidic chitinase gene (PR-3) was also strongly induced throughout the time course of defense response. Transcripts of this gene were increased in the mock- treated control, suggesting that this gene might be induced also by wounding, although not as quickly as by TMV-infection. Expression pattern of the phosphoglucomutase (PGM) gene was similar to that of the chitinase gene. The PGM gene was induced slightly by wounding and more strongly by TMV-infection. Expression of a proteinase inhibitor gene (clone 676) was induced either by mock- or TMV-treatment, but with higher degree expression was detected in the mock-treated leaves after 24 h after the treatment. It is well known that plants accumulate several classes of proteinase inhibitor in response to wounding and pathogen attack (Pautot et al., 1991).

The clone 215 showed high homology with known cysteine proteinase or Kunitz-type inhibitor genes. Expression pattern of this gene was quite different from other known

Table 1. Classification of cDNA clones selected from modified differential screening by database search

Classification	Numbers on slot blot	Sequenced and database searched	Classification		
			Defense-related genes	High score matched genes	Low score matched genes
Clone	900	81	25	41	15
Genes[a]		61	12	34	15

[a] subtracting the number of clones showing identical sequences.

Table 2. Blast analysis of cDNA clones selected from modified differential screening

No.[a]	R[b]	Expr.[c]	Putative Identification	Organisms	%LC[d]	%ID[e]	Acc. No.[f]
Defense-related genes							
59	1	+	Taumatin-like protein (PR-5)	*Nicotiana tabacum*	155	97	X15224
103	4		Glutathione-S-transferase	"	101	93	M29274
107	2	+	PR protein-1a (PR-1)	*N. glutinosa*	168	100	U49241
143	2		PR protein-4b (PR-4b)	*N. tabacum*	70	85	M60282
163	2	+	SAR8.2	"	95	91	M97361
198	2		Osmotin (PR-5)	"	116	99	X95308
239	3		Glycine-rich protein	"	86	99	D86721
260	1		Extensin-like protein	*Populus nigra*	59	72	D83226
358	4	+	Acidic chitinase (PR-3)	*N. tabacum*	143	95	M29868
512	1	–	Gamma thionin	"	96	87	Z11748
676	1	+	Proteinase Inhibitor type II	*N. alata*	151	79	U08219
Known and high score matched genes							
23	1		Proteosome subunit	*Lycopersicon esculentum*	101	97	Y14339
46	1		Ribosomal protein L36	*Daucus carota*	89	75	U47095
60	1	–	Plastocyanin	*L. esculentum*	139	69	X13934
63	1		PSI-D (photosystem I sub.II)	*N. sylvestris*	93	82	X60008
73	6		Rubisco SSU	"	124	91	X01722
94	1	+	Phosphoglucomutase	*Zea mays*	106	72	U89341
113	1		Glyceraldehyde3-p-dehydrogenase	*N. tabacum*	77	87	M14417
126	1		Cytochrome C reductase	*Solanium tuberosum*	69	78	X79275
138	1		Ferredoxin-I	*L. esculentum*	66	93	Z75520
156	1	–	Plastidic aldolase	*S. tuberosum*	67	95	Y10380
183	1		Kinesin homolog	*Arabidopsis thaliana*	99	74	Z97335
189	1		Potassium transporter	"	108	70	AF012660
190	2		Ribosomal protein S27	*Chalamydomonas reingardtii*	86	82	X83694
195	1		Cab binding protein 40	*N. tabacum*	193	92	X52744
197	1		Cab binding protein CP29	*Z. mays*	29	100	Z50801
207	1		Cyclophilin	*L. esculentum*	171	86	P21568
215	1	+	Proteinase inhibitor (putative)	*A. thaliana*	135	88	Z97343
218	1		40S Ribosomal protein S15	"	152	85	Z23161
227	1	–	Plastocyanin	*L. esculentum*	97	90	X13934
232	1		NADP-malate dehydrogenase	*Pisum sativum*	38	81	X74507
230	1		60S Ribosomal protein L37A	*Brassica. rapa*	81	93	L21897
342	1		Apospory-associated protein C	*Pennisetum ciliare*	113	69	D37938
406	1		14-3-3 protein	*N. tabacum*	116	99	Y11211
460	1		Replication factor C	*Homo sapiens*	114	66	M87339
478	1		Induced stolon tip protein	*S. tuberosum*	43	97	Z11679
506	1		Cab binding protein 36	*N. tabacum*	113	93	X58230
511	2		Metallothionein (type II)	*S. lycopersicum*	49	92	Z68310
545	1		PSII. water oxidizing comples	*N. tabacum*	75	97	X64349
580	1		Beta-amylase	*A. thaliana*	151	79	Z97342
603	1		Trios-phosphate translocator	*N. tabacum*	79	77	X75088
617	1		Zeta carotene desaturase	*Capsicum annuum*	65	66	U38550
669	1		Adenylate kinase	*Z. mays*	90	77	P43188
746	1		Cytochrome B6-F complex	*N. tabacum*	70	100	X66010
824	1		NAD-malate oxidoreductase	*S. tuberosum*	120	82	Z23023
Unknown and high score matched genes							
32	1		C25A1.6	*Caenorhabditis elegans*	59	57	Z81083
68	1		Y1bJ protein	*Bacillus subtilis*	37	43	Z98682
92	1		Suppressor of mitotic catastrophe	*Xenopus laevis*	52	34	U07608
193	1		Stromal cell-derived factor 2	*Mus musculus*	62	32	D50643
191	1		Ribosomal protein	*Brassica rapa*	34	47	D78495
239	1		Proline rich protein precursor	*Phaseolus vulgaris*	26	53	X60391
266	1		Gibberellin-regulated protein	*L. esculentum*	32	46	U53221
392	1		Hypothetical protein	*Synechocystis* sp.	65	44	D90913
414	1		Uridine kinase	*C. elegans*	60	50	Z69635

Table 2. Continued

No.[a]	R[b]	Expr.[c]	Putative Identification	Organisms	%LC[d]	%ID[e]	Acc. No.[f]
465	1		Outer arm dynein LC6	*Anthocidaris crassispina*	34	50	D1021360
473	1		F14E12.b	*C. elegans*	18	72	Z81060
581	1		Kinesin motor protein	*M. musculus*	56	30	U92949
605	1		NADH-ubiquinone oxidoreductase	*Apis mellifera ligustica*	25	48	L06178
630	1		Unknown	"			
672	1	+	Hypothetical protein	*Methanococcus jannaschii*	21	47	D17462

[a]No.: Clone number. All the clones have been stored in our laboratory and can be distributed upon request. [b]R. Redundency of the clone indicating the number of clones having identical nucleotide sequence. [c]Expr.: Induction (+) or repression (–) by TMV infection as observed in the modified differential screening. [d]LC: length compared indicating the number of amino acid residues between a query sequence and its matched protein sequence [e]%ID: % identity to the matched gene in amino acid sequence. [f]Accession number of the matched sequences.

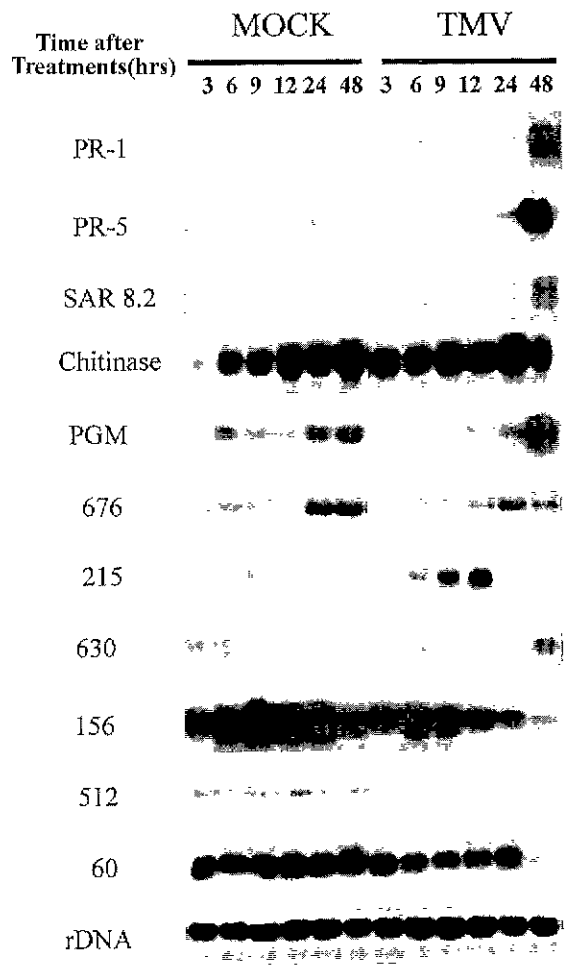

Fig. 3. Time course expression of some selected genes during defense responses against TMV infection. Total RNA was isolated from TMV- or mock-treated tobacco leaf tissues at the indicated time. RNA blots were hybridized with ^{32}P-labeled cDNA probes indicated in the figure.

proteinase inhibitor genes as shown with the clone 676. The clone 215 was rapidly and transiently induced 6 to 12 h after the TMV infection (Fig. 3). Thus, this gene might be involved in signal transduction pathway leading defense responses including HR. Recently, it was suggested that cystein proteases and protease inhibitor genes are involved in the regulation of HR, a form of programmed cell death in plants (D'Silva et al., 1998; Solomon et al., 1999). The possible function of the gene corresponding to the clone 215 in HR remains to be experimentally tested.

Upon the TMV-infection, expression of the genes was not always increased but decreased in some cases (Table 2). The clones 156 (plastidic aldolase), 512 (gamma thionin) and 60 (plastocyanin) were expressed in healthy (not shown) and mock-treated tobacco leaves (Fig. 3), suggesting that these genes are expressed constitutively. Upon TMV infection, transcripts of the genes were gradually reduced, exhibiting the lowest level at 48 h after the infection.

In summary, we have isolated genes differentially expressed during plant defense responses. In addition to the genes directly related to the defense responses, genes related to various primary and secondary metabolisms are induced or repressed upon pathogen attack. These results imply that defense response of plant against pathogen is a consequence of large changes in gene activity and complex reprogramming of cellular metabolisms. We are going to exert continuous efforts to contribute to understanding whole scheme of plant defense mechanism by performing more experiments aiming to identification of a complete set of defense-related genes.

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