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Comprehensive Analysis of Wound-inducible Genes from the *Nicotiana glutinosa* Leaves Using a Full-length cDNA Microarray

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Wound-inducible gene expression in the the *Nicotiana glutinosa* leaves was studied by using a microarray with 9600 full-length cDNAs. As a result, 86 genes were identified as wound-inducible genes in the *N. glutinosa* leaves, including those encoding defense related proteins, such as heat shock proteins, glutathione S-transferase, ascorbate peroxidase and non-specific lipid-transfer proteins. Among 86 genes, 15 genes including 11 hypothetical protein genes and 1 unknown protein genes encode unknown functional proteins. Although the translational products of these genes have not been characterized, they are potential candidates for defense-related proteins toward wounding. The cluster analysis classified the genes into 6 groups on the basis of their expression patterns. It is likely that genes clustered in the same groups may be co-regulated by common transcriptional factors and also translational products belonging to the same clusters may share common functions in defense response to wounding.

INTRODUCTION

Since plants are continuously exposed to various environmental and biological stresses, they have evolved a number of mechanisms to cope with different biotic and abiotic stresses. Among stresses, a mechanical wounding followed by pathogen attacks is a continual threat to the survival of plants. Once wounded and pathogen invaded, plants induce various defense-related gene expressions in a time window between a few minutes to several hours to produce the substances that restore damaged tissues and also inhibit growth of pathogens. The first identified wound-inducible defense proteins are proteinase inhibitors I and II from potato and tomato (Graham *et al.*, 1986; Ryan, 1990). To date, a number of defense-related genes have been identified in various plants; some of these defense-related genes encode transcription factors, osmotin, and several enzymes, such as proteases, chitinases, and peroxidases (Reymond and Farmer, 1998; De Bruxelles and Roberts, 2001; Jameson and Clarke, 2002). Although a vast amount of information about defense-related genes as well as chemical signals for a signal transduction have accumulated for diverse plant species, a detailed network of defense-related genes in a

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give plant has not been elucidated. To this end, it is essential to be aware of extensive changes of the gene expression that occur in a given plant under wounding and pathogen attacks.

The DNA microarray has recently been used to monitor a global gene expression in response to several stresses in higher plants. In the analysis, more than 10000 genes of interest organism could be simultaneously analyzed in terms of their expression profiles. In *Arabidopsis*, Seki *et al.* (2001; 2002) monitored expression of genes in response to cold, drought, and salt stress. Gong *et al.* (2001) used 84 salt-regulated cDNAs to profile transcription of wild type and the salt-hypersensitive mutant *sos3* and Fowler and Thomashow (2002) profiled transcripts responding to cold acclimation. In rice (Dubouzet *et al.*, 2003) and barley (Oztur *et al.*, 2002), cDNA microarray was used to study transcriptional profiling in response to salt and drought stress. In maize kernels and immature ears, Zinselmeier *et al.* (2002) used cDNA microarrays to monitor expression of 384 genes in response to shade stress and used oligonucleotide microarrays to examine expression of 1,502 genes in response to water stress. These studies have provided new insights into gene expression involved in stress responses at a genomic level and are contributing to understanding of functions of the responding genes. Furthermore, the cDNA microarray analysis is a useful method for efficiently exploring the functions of uncharacterized genes in addition to known genes by relating the expression pattern of one gene to those of others.

Nicotiana glutinosa is a diploid tobacco plant and displays TMV-resistance mediated by *N*-gene. The *N*-gene, cloned from tobacco, is a member of the Toll-IL-1 receptor homology region (TIR)-nucleotide binding site (NBS)-leucine-rich repeat region (LRR) class of *R* genes and confers resistance to the viral pathogen TMV (Whitham *et al.*, 1994). That is, TMV-infection at 25 °C causes hypersensitive cell death in leaves. In contrast, when *N. glutinosa* infected with TMV was kept at 35 °C, no local lesions were formed on the leaves, but systemic infection occurred. These observations led us to the expectation that comparison of the gene expression in response to TMV-infection at 25 °C and 35 °C would provide essential genes responsible for TMV resistance of *N. glutinosa*. For an initial step for this study, we have prepared the *N. glutinosa* full-length cDNA microarray containing 9600 cDNAs and first studied gene expression dynamics in mock-inoculated (mechanical wounding) *N. glutinosa* leaves. In this paper, we described identification and the cluster analysis of wound-inducible genes in the *N. glutinosa* leaves.

MATERIALS AND METHODS

Plant material, stress treatments and RNA isolation.

The plants (*N. glutinosa*) were germinated and grown in soil pots in a greenhouse of Kyushu University under natural lighting at 25 °C for 1.5 months. The *N. glutinosa* leaves were dusted with Carborundum (600 mesh) and rubbed with cotton pads moistened with 100 mM Na-phosphate buffer, pH 7.2, in the absence (mock-infection or wounding) or presence of TMV (10 μg/ml) (TMV-infection). After 0.5 h, 24 h, and 48 h, leaves were harvested, frozen in liquid nitrogen, and stored at −80 °C until use. The total leaf RNA was extracted by the method of Shirzadegan *et al.* (1991)

Preparation of full-length cDNA library.

The oligo-capping cDNA was prepared by the method of Suzuki and Sugano (2001), as follows. Ten μg of poly(A)$^+$ RNA purified by utilizing Oligotex™-dT30 <Super> (TAKARA BIO) from *N. glutinosa* leaves were treated with 1.2 unit of bacterial alkaline phosphatase (BAP: TAKARA BIO) in 220 μl of 100 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol with 100 unit of RNasein (Promega) at 37 °C for 40 min. After extraction with phenol/chloroform/isoamylalcol (24/25/1) twice and ethanol precipitation, the poly(A)$^+$ RNA was treated with 20 units of tobacco acid pyrophosphatase (TAP: Nippon gene) in 100 ml of 50 mM sodium acetate (pH 5.5), 1 mM EDTA, 5 mM 2-mercaptoethanol with 100 units of RNasein at 37 °C for 45 min. After extraction with phenol/chloroform/isoamylalcol (24/25/1) and ethanol precipitation, the poly(A)$^+$ RNA (4~6 μg) treated with BAP and TAP were ligated with 0.5 mg of RNA oligonucleotide, 5'-GAG ACG GAU CCU AAA CAA UUA ACC CUC AAA-3' using 50 units of T_4 RNA ligase (TAKARA BIO) in 100 μl of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.5 mM ATP, 25% PEG8000 with 100 units of RNasein at 20 °C for 16 h. After removing unligated RNA oligonucleotide by extraction with phenol/chloroform/isoamylalcol and ethanol precipitation, first-stranded cDNA was synthesized with AMV reverse transcriptase using 5'-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T-3' in 50 μl with 2~4 μg of oligo-capped poly (A)$^+$RNA at 42 °C for 60 min. After first strand synthesis, RNA was degradated in 15 mM NaOH by incubating at 65 °C for 60 min. The cDNA made from 0.7 mg of oligo-capped poly (A)$^+$ RNA was amplified by PCR with Ex *Taq* polymerase (TAKARA BIO) using forward primer, 5'-GAG ACG GAT CCT AAA CAA TTA ACC CTC AAA-3' and back primer, 5'-GGC CAC GCG TCG ACT AGT AC-3'. Amplification cycles were 10 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 10 min. The PCR products were separated by an agarose gel electrophoresis and those longer than 700 bp were isolated and ligated into the pGEM™-T Easy vector (Promega). The ligation mixtures thus obtained were stored at -80 °C until use. For cDNA cloning, the mixtures were used for transformation into *E. coli* JM109 strain.

Amplification of cDNA insert.

Discretionary cDNAs (9600 clones) were grown in twenty five 384-well microtiter plates at 37 °C for 16 h. The cDNAs were amplified by PCR using cultures as template and same primers as double-stranded cDNA amplification. Amplifications were done for 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The PCR products were precipitated in 2-propnanol, then resuspended at ~2 mg/ml in TE buffer, mixing 20 times using Multimek 96/384 Multi-Channel Pipettor (Beckman Coulter).

Microarray preparation.

The PCR products were arrayed from 384-well microtiterplate onto a microslide glass (Matsunami, Japan) using the microarray stamping machine SPBIO (Hitachi software Engineering Co., Ltd., Japan). The printed slides were dried and subjected to UV cross-linking (600 mJ×100). The slides were rocked in 70 mM succinic anhydride/0.1 M borate buffer dissolved in 1-methyl-2-pyrrolidone for 30 min and then rocked in Milli-Q water for 30 s thrice vigorously. The slides were transferred into the boiling Milli-Q water for 3 min and then ice-cold ethanol for 5 min. The slides were air-dried and used for

further hybridization step.

Preparation of probes.

Total RNAs from healthy leaves or treated leaves (10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, and 72 h after treatment) were reverse-transcribed using Powerscript (Clontech) in the presence of amino-allyl dUTP (Sigma). Each reaction was performed in a 40 μl volume containing 50 μg of total RNA, 2 μg of oligo (dT) 18 mer (New England Biolabs), 0.5 mM each for dATP, dCTP, dGTP, and 0.2 mM dTTP, 0.3 mM amino-allyl dUTP (Sigma), 10 units of RNasein, 10 mM DTT, and 2 μl of Powerscript reverse-transcriptase in Powerscript first-strand buffer. After incubation at 42 °C for 1 h, 2 μl of Powerscript was added and the mixture was further incubated for 1 h. The reaction was stopped by addition of 50 μl of 100 mM EDTA and RNA in the reaction mixture was degraded by adding 20 μl of 1 M NaOH at 60 °C for 30 min. The reaction mixture was neutralized by addition of 50 μl of 1 M Tris-HCl (pH 7.5). The synthesized cDNA was purified by size-exclusion spin column (microcon30, Amicon) and dissolved in 20 μl of 50 mM $NaHCO_3$. Labeling reactions with Cy5 monofunctional dye (Amersham Biosciences) for control samples (healthy leaves) or Cy3 monofunctional dye for treated samples (wounded leaves) were performed in the dark at room temperature for 60 min. After blocking by NH_2OH, the Cy3-labeled and Cy5-labeled probes were pooled in a same tube and incubated at room temperature for 15 min. The excess reagents were removed by PCR purification kit (Qiagen). The labeling cDNA probes were checked by an agarose gel electrophoresis and used for hybridization.

Hybridization reaction and microarray analysis.

Before hybridization, the cDNA solution (40 μl) containing 5×SSC and 0.5% SDS was heated at 65 °C and then used for microarray analysis. The microarrays were placed in hybridization cassette and Milli-Q water (7 μl) was placed inside each chamber before sealing and then incubated for 10~20 h at 60 °C. After incubation, the microarrays were sequentially washed thrice for 5 min in 2×SSC-0.2% SDS, thrice for 5 min in 0.2× SSC-0.2% SDS, thrice at 60 °C for 20 min in 0.2×SSC-0.2% SDS, thrice for 5 min in 0.2× SSC-0.2% SDS, four times for 10 s in 0.2×SSC, and finally twice in 100% ethanol. Microarrays were dried by centrifugation at 800 rpm for 3 min, and then scanned with Bio-imaging analyzer BAS-5000 (Fuji Film). The image files were analyzed using Arrayvison software (Imaging Research Inc.).

Data analysis.

A difference in fluorescent strength between Cy3 and Cy5 causes a bias of the expression quantify ratio. The signal intensities of duplicate spots on an array were therefore normalized by locally weighted linear regression analysis (LOWESS) (Shirzadegan *et al.*, 1991). Then, the average intensity ratio (Cy3/Cy5) followed by $\log_2$ (Cy3/Cy5) was calculated. Furthermore, cDNA clones showing a signal value lower than 10000 in both Cy3 and Cy5 channels were eliminated for analyses. For screening wound-inducible genes, the median and standard deviation (SD) of $\log_2$Cy3/Cy5 were calculated, and then, the cDNA clones with expression ratio ($\log_2$Cy3/Cy5) greater than median + 2SD in at least one time-course point were selected as wound-inducible genes.

All of the processed data then were subjected to the self-organizing map algorithm followed by complete linkage hierarchical clustering of microarray genes, as described by Eisen *et al.* (1998) We used the default options of hierarchical clustering using the uncentred correlation similarity metric.

cDNA sequencing analysis for microarray.

The nucleotide sequences of the cDNAs selected as wound-inducible genes were determined using the dye terminator cycle sequencing method (CEQ2000 Dye Terminator Cycle Sequencing with Quick Start Kit; Beckman Coulter) with a DNA sequencer CEQ2000XL (Beckman Coulter). Homology search was performed with the Gen-Bank/EMBL database using the BLAST program (Julich, 1995).

Validation of microarray data by reverse transcription PCR.

The expression patterns of wound-inducible genes were confirmed by reverse transcription PCR (RT-PCR). The RT-PCR procedure was done as follows. Total RNAs (1 μg) extracted from wound treatment leaves (30 min, 2 h, 8 h, 24 h and 48 h after treatment) were reverse-transcribed using Powerscript (Clontech) with oligo d(T) 18 primer (Biolabs). The cDNA mixture was amplified by LA-*Taq* (TAKARA BIO) with the following gene-specific primer pairs: NgA5004-forward primer, 5'-CATATGGGAGGAGGAACAGAAGCTTTTCCA-3'; NgA5004-reverse primer, 5'-CTCGAGACAAGCTTTAACGGAAGGGGTGGT-3'; NgC6193-forward primer, 5'-ATGGAATTGGCTGGCAAGATTGCATGTTTT-3'; NgC6193-reverse primer, 5'-CTGGACCTTGGACCAGTCAGTGGAGGGGCT-3'; NgB1164-forward primer, 5'-TGTGGGTGCTGTTGGGCATTTTCTGCAGTA-3'; NgB1164-reverse primer, 5'-ATCTATTGCCACCGAAACAGGTTGATTAGC-3'; rRNA-forward primer, 5'-TCTCGGCTCTCGCATCGATGAAGAACGTAG-3'; rRNA-reverse primer, 5'-GCGGGCGGGGGCGACGCGATGCGTGACGCC-3'. The samples were first denatured by heating at 94 °C for 2 min and then incubated for 25 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min. The samples were finally incubated for 5 min at 72 °C. The amplified products were analyzed by 1% agarose gel electrophoresis.

RESULTS AND DISCUSSION

Preparation of the *N. glutinosa* cDNA Microarrray

We constructed full-length cDNA libraries from the *N. glutinosa* leaves which were non-treated or wounded followed by TMV-infection, as described under Materials and methods. We unintentionally selected 9600 cDNA clones, amplified them by PCR and arrayed onto glass slides, as described under Material and methods. Judging from the sequence analyses of 100 cDNA clones, we estimated that approximately 4000 unique clones were included in the selected cDNA clones. The average frequency of full-length cDNA clones in the library was about 50%.

In order to evaluate the cDNA microarray as well as the method for data normalization, the total RNA extracted from wounded leaves was split in two identical aliquots, to exemplify mRNAs from control and treated leaves. Two populations of single stranded cDNAs were generated from the two aliquots and labeled with Cy3 and Cy5 fluorophores,

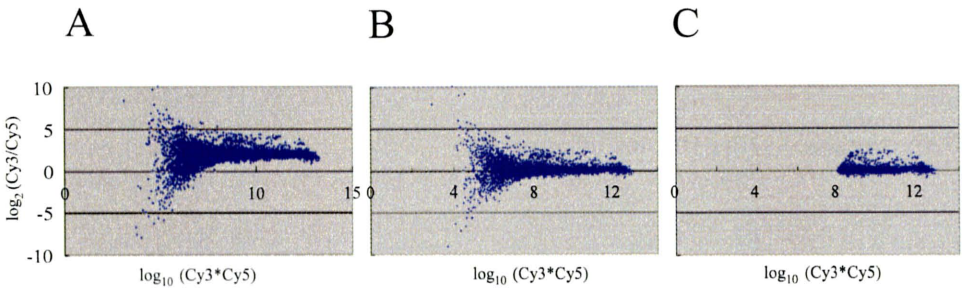

Fig. 1. Normalization of the Data obtained by Microarray analysis

A, An R–I plot displays the $\log_2$ (Cy3/Cy5) ratio for each element on the array as a function of the $\log_{10}$ (Cy3*Cy5) product intensities and can reveal systematic intensity–dependent effects in the measured $\log_2$ (Cy3/Cy5) values. B, Application of local lowess can correct for both systematic variation as a function of intensity and spatial variation between spotting pens on a cDNA microarray. C, Normalization data were obtained by elimination of the signal values lower than 10,000 of genes ($\log_{10}$ Cy3/Cy5 < 8).

respectively. The two samples of labeled cDNA were simultaneously hybridized to the microarray, and the data were evaluated as described under Materials and methods. In this experiment, each transcript is equally abundant in the two samples. Hence a $\log_2$Cy3/Cy5 signal ratio of 0 should ideally result for each spot in the microarray. As given in Fig. 1A, the R–I plot calculated with law data shows a slight shift of signal intensity ratios between Cy3 and Cy5 from x axis. This result suggested different fluorescent strengths between Cy3 and Cy5 or different background levels of cDNAs dependent on the spotting positions in the microarray. Then, all data were normalized by LOWESS so that signal ratios of almost all data were replicated to x axis (Fig. 1B). In addition, we eliminated data whose signal values were lower than 10,000 ($\log_{10}$Cy3/Cy5<8). As a result, almost all data were approximate around the x axis, indicating the dependability of the *N. glutinosa* cDNA microarrray for further analyses by normalization and elimination of low fluorescence intensity clones (Fig. 1C).

Screening and Identification of Wound– inducible Genes

For studying of a temporal program of transcription occurred in the *N. glutinosa* leaves in response to wounding, the *N. glutinosa* leaves at ten time points (10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, and 72 h) up to 72 h after wound–treatment were detached and mRNA was purified therefrom. The cDNA made from each sample was labeled with the fluorescent dye Cy3 and mixed with a reference probe, consisting of cDNA made from healthy plants and labeled with the second fluorescent dye, Cy5. The two populations of labeled cDNAs were simultaneously hybridized with the cDNA microarray. Then, they were scanned by two separate laser channels for Cy3 and Cy5 emissions from DNA elements. After scanning fluors, the signal intensity for each cDNA was integrated to calculate the ratios of the fluorescence intensities of two probes. We performed ten separate hybridizations to monitor changes in transcripts in comparison

with those of healthy plants at ten time points. To assess the reproducibility of the microarray analysis, the hybridizations were performed twice. The hybridization of different microarrays with the same mRNA samples showed a good correlation. A pseudocolor image of the results obtained for one time point (60 min after wound–treatment) is shown in Fig. 2.

All microarrays were normalized and cDNAs showing signal values lower than 10000 were eliminated. A typical scatter plot of signal intensities and its normalization are presented in Fig. 3. The median and SD of the signal ratios were calculated for spots on each glass slide. The wound–inducible genes were selected from ten time points of microarray, described in Materials and methods, resulting in that 104 clones were selected for further study.

These cDNAs were partially sequenced and the sequence data were analyzed with the BLAST program. The results are summarized as given in Table 1. Eleven wound–inducible genes occurred in more than two clones; the gene encoding the homologue of chroloplast thiazole biosynthetic protein from *Nicotiana tabacum* (LaFayette *et al.*, 1996) was the most abundant gene identified: 8 clones possessed the identical gene. In this analysis, 11 and 1 genes whose sequences had sequence similarities to those of hypothetical protein genes and unknown protein genes, respectively, were identified as wound–inducible genes. Further biochemical characterization of their translational products will define their functions in plant defense. As a result, 86 independent genes were identified as wound–inducible genes. The DNA sequences of all

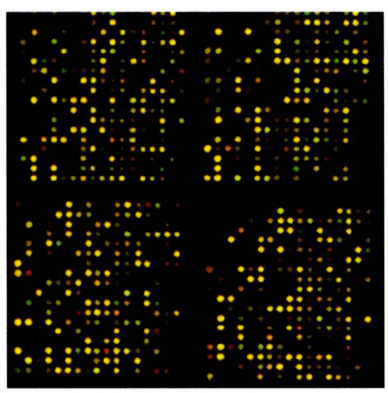

Fig. 2. Microarray Analysis of Gene Expression after Mechanical Wounding

A fluorescently labeled cDNA probe was prepared from mRNA isolated from control *N. glutionosa* leaves by reverse transcription in the presence of Cy3–dCTP. A second probe, labeled with Cy5–dCTP, was prepared from leaves that were mechanically wounded (60 min). After the simultaneous hybridization of both probes with cDNA microarray containing 9600 cDNA clones and scanning of the array, a pseudocolor image was generated. Genes induced or repressed after mechanical wounding are represented as green or red signals, respectively. Genes expressed at approximately equal levels between treatments appear as yellow spots. The intensity of each spot corresponds to the absolute amount of expression of each gene.

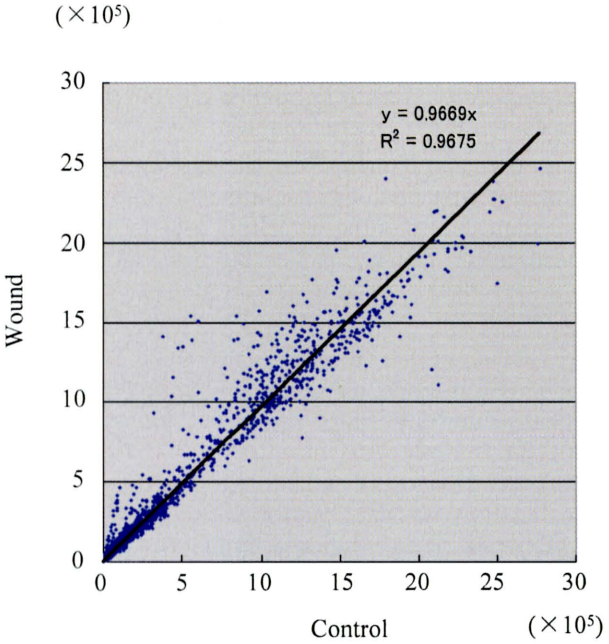

Fig. 3. A scatter plot of signal intensities for cDNAs on the microarray
Normalized channel intensities for each cDNA clone on the microarray are plotted with signals from control and wounding on the x and y axes, respectively.

genes are listed at http://seika.ath.cx/cgi/tabako/.

Confirmation of Gene Expression by Reverse Transcription PCR

To corroborate the result obtained by the microarray analysis, RT–PCR was done for several selected genes using specific primers. Figure 4A shows representative results of RT–PCR for three wound–inducible genes. The transcripts of the gene NgA5004 began to accumulate 30 min after wounding, reached a maximum level within 2 h, and then disappeared. The expression of the gene NgC6193 was significantly induced by wounding within 24 h and this increase was observed through 48 h. In contrast, the expression of the gene NgB1164 was induced twice at 2 h and 24 h after wounding. These results were in a good agreement with the results obtained by the microarray analysis (Fig. 4B). Similar results were obtained for other selected genes (data not shown), demonstrating characteristic expression patterns of wound–inducible genes derived from the microarray analysis.

Cluster Analysis of the Wound–inducible Genes

We did cluster analysis to classify the 104 wound–inducible genes derived from the microarray experiment according to patterns of their gene expression, as described under

Table 1. Wound–inducible Genes in the *N. glutinosa* Leaves.

Gene number	Homologue	Accession number	Source	Homologue in Arabidopsis	Enzyme category[a]	Frequency
NgB2261	Molecular chaperone Hsp90-2	AY368905	*Nicotiana benthamiana*	BT000717	NE	3
NgC5246	Molecular chaperone Hsp90-1	AY368906	*Lycopersicon esculentum*	AY368906	NE	1
NgC5076	Heat shock protein 90	AB111810	*Oryza sativa*	At5g52640	NE	1
NgA8254	Heat shock protein 90	AY519499	*Nicotiana tabacum*	AY062832	NE	1
NgB6279	Heat shock protein 90 homologue T22A6.20	T09882	*Arabidopsis thaliana*	At4g24190	NE	1
NgB6252	Heat shock protein 70	AJ249330	*Cucumis sativus*	At3g12580	NE	1
NgC4267	Heat shock protein 70	AY253326	*Nicotiana tabacum*	At3g12580	NE	1
NgD1263	Heat shock 70 protein	X13301	*Petunia x hybrida*	At5g02490	NE	1
NgA6164	70 kDa heat shock protein	AF479058	*Sandersonia aurantiaca*	AY035123	NE	1
NgC8014	Cytosolic heat shock 70 protein	AF033852	*Spinacia oleracea*	At3g12580	NE	1
NgD4230	DnaK-type molecular chaperone hsc70-3	Q40151	*Lycopersicon esculentum*	AC069474	NE	1
NgB4166	DnaK-type molecular chaperone hsc70-3	L41253	*Lycopersicon esculentum*	At3g12580	NE	2
NgD7292	DNAJ protein	AJ299254	*Nicotiana tabacum*	At3g44110	NE	1
NgA5040	Heat shock cognate 70 kDa protein	X54030	*Lycopersicon esculentum*	At3g12580	NE	1
NgA1020	APG protein	AY114586	*Arabidopsis thaliana*	AY114586	Hy	1
NgB5184	Nonspecific lipid-transfer protein 2 precursor	Q03461	*Nicotiana tabacum*	At2g38540	NE	1
NgC6193	Nonspecific lipid-transfer protein 2 precursor	D13952	*Nicotiana tabacum*	At2g38540	NE	1
NgD8196	Nonspecific lipid-transfer protein 2	S29227	*Nicotiana tabacum*	At2g38540	NE	1
NgB5071	Non-specific lipid transfer protein	AF525363	*Solanum tuberosum*	At2g38540	NE	1
NgB1164	Cysteine protease	AF242372	*Ipomoea batatas*	At5g45890	Hy	1
NgC7073	Cytosolic ascorbate peroxidase	D85912	*Nicotiana tabacum*	X59600	Ox	2
NgA5242	Ascorbate peroxidase	U15933	*Nicotiana tabacum*	At4g35000	Ox	2
NgD7272	Aquaporin-like protein	AF452015	*Petunia x hybrida*	AY087558	NE	2
NgA6248	Glutathione S-transferase T3	AY007560	*Lycopersicon esculentum*	At2g29420	Tr	1
NgA4284	DNA-binding protein tbz17	D63951	*Nicotiana tabacum*	AF400620	NE	1
NgC1079	MYB transcription factor	AY519516	*Arabidopsis thaliana*	AY519516	NE	1
NgA5004	Zinc finger protein	AF139098	*Arabidopsis thaliana*	AF139098	NE	1
NgB8251	Ethylene-responsive transcriptional coactivator	AF096246	*Lycopersicon esculentum*	At3g24500	NE	1
NgA7295	Ethylene-forming enzyme EFE	S41395	*Nicotiana tabacum*	At1g05010	Ox	1
NgD3254	GTP-binding protein	AP002524	*Oryza sativa*	At1g02170	NE	1
NgB3046	Calnexin precursor	U20502	*Glycine max*	At5g07340	NE	2
NgA8136	Thioredoxin H-type 1	X58527	*Nicotiana tabacum*	At1g19730	Ox	2
NgB3142	Abscisic stress ripening protein 2	X74907	*Lycopersicon esculentum*	No homolog	NE	1
NgA3264	Ribulose bisphosphate carboxylase small subunit protein	AY220079	*Nicotiana tabacum*	At5g38410	Ly	2
NgA7246	RuBisCO subunit binding-protein beta subunit, chloroplast precursor	M35600	*Brassica napus*	AC005223	Ly	1
NgA7149	ADP,ATP carrier protein, mitochondrial precursor	X62123	*Solanum tuberosum*	AY042814	NE	1
NgB1266	Chlorophyll a-b binding protein 2, chloroplast precursor	X12735	*Hordeum vulgare*	At2g05100	NE	1
NgB8271	Chlorophyll a/b-binding protein type I	X64198	*Nicotiana tabacum*	AY085893	NE	1
NgA5295	Chloroplast thiazole biosynthetic protein	AY220080	*Nicotiana tabacum*	At5g54770	NE	8
NgB1136	Phi-1 protein	AB018441	*Nicotiana tabacum*	AC079605	NE	2
NgD4215	Glutamate decarboxylase	AF020424	*Nicotiana tabacum*	At2g02010	Ly	1
NgD3297	Glutamate decarboxylase	AF506366	*Nicotiana tabacum*	At2g02000	Ly	1
NgA6124	RNA-binding glycine-rich protein-1	D16206	*Nicotiana sylvestris*	S30148	NE	1
NgB8144	Glycine-rich RNA-binding protein	D16204	*Nicotiana sylvestris*	AC007119	NE	1
NgC6050	Plastidic ATP/ADP-transporter	Y10821	*Solanum tuberosum*	At1g80300	NE	1
NgC2240	Protein transport protein SEC61 alpha subunit	AY093047	*Arabidopsis thaliana*	AY093047	NE	1
NgC6111	Luminal binding protein 4 precursor	JQ1360	*Nicotiana tabacum*	At5g42020	NE	1
NgC8270	Luminal binding protein 5 precursor	Q03685	*Nicotiana tabacum*	At5g42150	NE	1
NgD4101	Thiosin like protein	AB034956	*Nicotiana tabacum*	AAL85136	NE	1
NgC5160	SUMO E2 conjugating enzyme SCE1	AJ580839	*Nicotiana benthamiana*	AL132977	Lg	1
NgC4281	Anthocyanidin 3-O-glucoside-6"-O-malonyltransferase	AF489109	*Dahlia variabilis*	At3g29577	Tr	1
NgB5189	GDSL-like lipase/acylhydrolase	AC128645	*Oryza sativa*	AY072081	Hy	1
NgC3169	Cell elongation protein diminuto	AE017090	*Oryza sativa*	AB025631	NE	1
NgA4227	Acid phosphatase	AJ250282	*Hordeum vulgare subsp. vulgare*	AL035523	Hy	1
NgA7171	Ribosomal protein L34	AC021043	*Arabidopsis thaliana*	AC021043	NE	1
NgA3237	Retrotelement pol polyprotein	AC004483	*Arabidopsis thaliana*	AC004483	Tr/Hy	1
NgD8255	Phospholipid hydroperoxide glutathione peroxidase	Q9FXS3	*Nicotiana tabacum*	At4g11600	Ox	1
NgA7120	Epoxide hydrolase	Q41413	*Solanum tuberosum*	At4g02340	Hy	1
NgB5192	NADP-specific isocitrate dehydrogenase	AB109115	*Lupinus albus*	AJ437268	Ox	1
NgC6047	Malate dehydrogenase	AF001270	*Lycopersicon esculentum*	At5g11670	Ox	1
NgB7244	Lethal leaf spot 1-like protein	AF321984	*Lycopersicon esculentum*	U77347	NE	1
NgD8190	Glyceraldehyde 3-phosphate dehydrogenase	AF527779	*Solanum tuberosum*	At1g42440	Ox	1
NgB3167	Glucosyltransferase IS5a	T03747	*Nicotiana tabacum*	At4g34120	Tr	1
NgD8124	EEF13 protein	AB032753	*Solanum melongena*	At4g29270	Hy	1
NgD1191	Caffeic acid O-methyltransferase II	AF484252	*Nicotiana tabacum*	AB013387	Tr	1
NgC8247	C-8,7 sterol isomerase	At1g20050	*Arabidopsis thaliana*	At1g20050	Is	1
NgA2123	Alpha-tubulin	AJ421411	*Nicotiana tabacum*	AL161540	NE	1
NgA5299	Alpha-glucan phosphorylase, H isozyme	A40995	*Solanum tuberosum*	At3g46970	Tr	1
NgC4236	Adenosine kinase-like protein	AY224510	*Oryza sativa*	At3g09820	Tr	1
NgB8193	Acyl-CoA synthetase-like protein	AF503771	*Arabidopsis thaliana*	AF503771	Lg	1
NgC7214	24K germin like protein precursor	AB112080	*Nicotiana tabacum*	AY081576	Hy	1
NgB2106	21K protein precursor	Y11553	*Medicago sativa subsp. x varia*	AL096860	NE	1
NgB8153	Water-stress inducible protein	AF010584	*Oryza sativa*	No homolog	NE	1
NgC7273	Stress-related protein	U54704	*Phaseolus vulgaris*	At3g05500	NE	1
NgC2181	Unknown protein	AY035092	*Arabidopsis thaliana*	AY035092		1
NgA8245	Hypothetical protein	At3g15840	*Arabidopsis thaliana*	At3g15840		1
NgB3054	Hypothetical protein	At2g01100	*Arabidopsis thaliana*	At2g01100		1
NgB4277	Hypothetical protein	AL078467	*Arabidopsis thaliana*	AL078467		1
NgB5153	Hypothetical protein	At2g38180	*Arabidopsis thaliana*	At2g38180		1
NgB7039	Hypothetical protein	AL021889	*Arabidopsis thaliana*	AL021889		1
NgC2179	Hypothetical protein	AC005170	*Arabidopsis thaliana*	AC005170		1
NgC7035	Hypothetical protein	At1g12440	*Arabidopsis thaliana*	At1g12440		2
NgB4298	Hypothetical protein	AX647765	*Homo sapiens*	At2g01140		1
NgB5257	Hypothetical protein	AL731629	*Oryza sativa*	AY081836		1
NgB6265	Hypothetical protein	At4g22820	*Arabidopsis thaliana*	At4g22820		1
NgA2070	Hypothetical protein	At4g12040	*Arabidopsis thaliana*	At4g12040		1

[a] NE, non-enzyme; Ox, oxidoreductase; Tr, transferase; Hy, hydrolase; Ly, lyase; Is, isomerase; Lg, ligase.

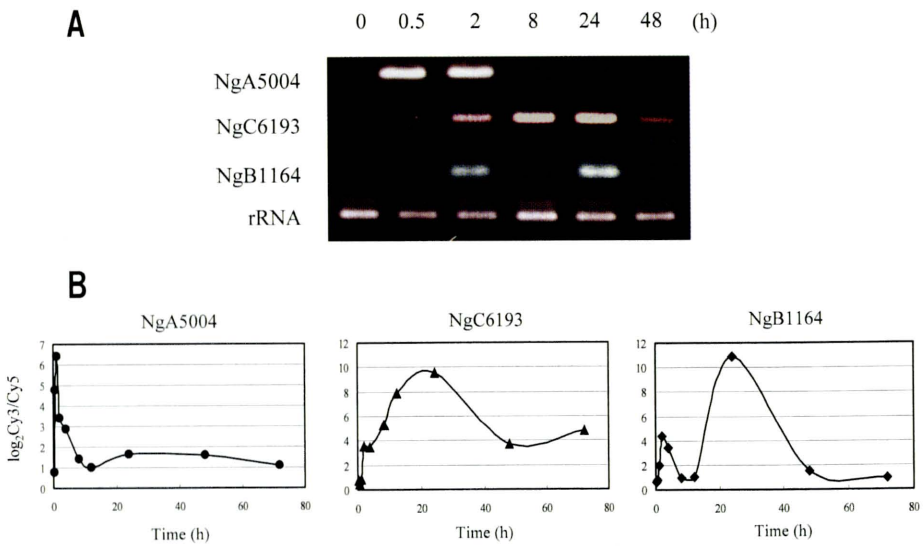

Fig. 4. Comparison of the microarray data and RT–PCR analysis for three selected genes
A, Total RNA from the *N. glutinosa* leaves at 0.5, 2, 8, 24, and 48 h after wounding were extracted and amplified, as described under Materials and methods. The 18S rRNA at the bottom was done to serve as a total RNA control. B, Expression patterns of the three selected genes described from the microarray analysis were shown.

Materials and methods. Figure 5 illustrates a hierarchical clustergram of genes which were classified into 6 groups by related regulation patterns (correlation values <0.85). Obviously, it could be expected that genes identified by more than two clones are clustered in the same groups. For example, 8 putative chloroplast thiazole biosynthetic protein and 4 nsLTPs were perfectly classified into the same groups II and I, respectively. This result demonstrates the experimental reliability of our data obtained by the microarray analysis.

It is generally supposed that the early responsive genes are involved in a signal transduction for a specific expression of defense–related genes of plants. The transcripts of genes belonging to group VI began to accumulate as early as 10 min after wounding and disappeared after 1 h. In addition, the transcripts of the genes included in groups V and IV began to accumulate 30 min after wounding, reached a maximum level within 1 h and gradually decreased during 4 h and 8 h. Groups VI and V include several hypothetical protein genes. Although their translational products have not been characterized, they may play a role in a signal transduction for lately inducible expression of genes. In contrast to the early responsive expression of the genes included in groups VI and IV, the transcripts of the genes belonging to groups III, I, and II began to accumulate 4 h after wounding, and therefore these genes are considered to be late–responsive genes. These genes include those encoding heat shock proteins, non–specific lipid transfer proteins, and several enzymes, such as adenosine kinase and glutathione S–transferases. These

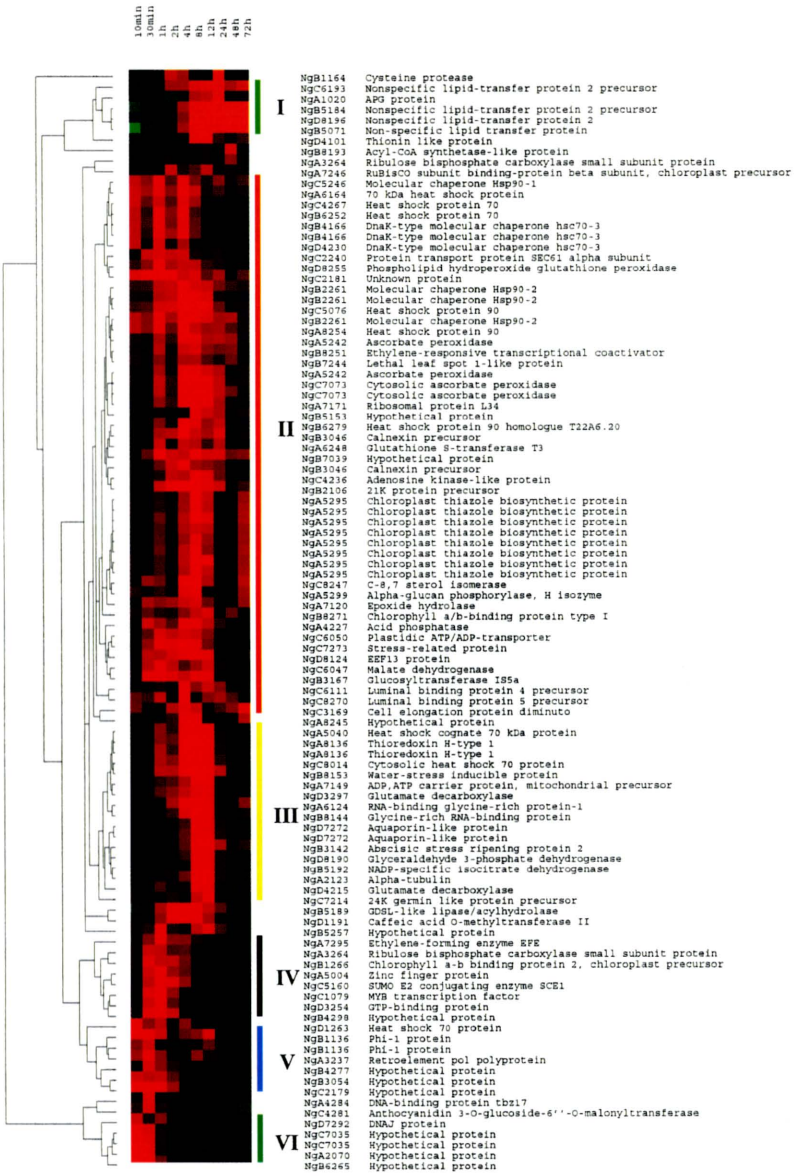

Fig. 5. Clustered display of data from the time course of mechanical wounding

A time course of wound–inducible gene expression in the *N. glutinosa* leaves was constructed using cDNA microarrays. For simplicity, only those genes for which the transcript levels changed substantially as a result of wounding are included. Genes were ordered using a clustering program (see Materials and methods) so that those with similar expression patterns would be grouped together. Each gene is represented by a single row of colored boxes, and each time point is represented by a single column. Induction (or repression) ranges from pale to saturated red (or green).

translational products may serve as effecter proteins or enzymes in metabolisms or plant defense. A characteristic feature of the genes included in group II was to be induced twice in response to wounding. The first transcripts began to accumulate 1 h after wounding and decreased within 2 h. Then, the second induction occurred and the transcripts reached a maximum at 4 h after wounding. This group predominantly includes genes encoding either heat shock proteins or molecular chaperones. Their translational products may play dual functions in defense reaction of the *N. glutinosa* leaves. In general, it could be expected that genes clustered in the same groups may be co-regulated by common transcriptional factors and also translational products belonging to the same clusters may share similar or related roles in defense toward wounding. Hence, further studies on the structure of the genes and their translational products will provide valuable insight into the molecular mechanism of defense response to wounding.

Possible Translational Products

In the present study, we screened wound-inducible genes in the *N. glutinosa* leaves by using cDNA microarray, and 86 genes were assigned as wound-inducible genes. Their possible translational products include 30 enzymes and 45 non-enzyme proteins, as given in Table 1. Among the enzymes, there are 8 oxidoreductases, 7 transferases, 8 hydrolases, 4 lyases, one isomerase and 2 ligases. It is known that cellular metabolism is altered by environmental stresses. For example, scavenger enzymes such as glutathione S-transferase and ascorbate peroxidase function as reactive oxygen species (ROS) elimination in condition of oxidative stress (Wagner *et al.*, 2002; Yoshimura *et al.*, 2000). Furthermore, several kinases and phosphatases are known to be involved in a signaling pathway for responsive induction of defense-related genes (Asai *et al.*, 2002; Hailling *et al.*, 2003). However, detailed analyses on the effects of stress on the majority of the enzymes in individual metabolic pathways are lacking. Hence, a high throughput analysis of the enzymes would lead to a better understanding of their defensive role in response to wounding.

As for non-enzyme proteins, many genes encoding heat shock proteins (HSPs) and molecular chaperones were identified as wound-inducible gene in the *N. glutinosa* leaves. While HSPs ostensibly function in the development of thermotolerance, their expression levels would be increased when the plant is subjected to other stresses such as water stress, heavy-metal toxicity and cold stress (Coca *et al.*, 1996; Gyorgyey *et al.*, 1991; Sabehat *et al.*, 1998). Beside environmental stresses, HSP in plants is also synthesized in certain stages of development such as embryogenesis, germination, pollen development and fruit maturation (Gyorgyey *et al.*, 1991; DeRocher and Vierling, 1995; Laurence *et al.*, 1997; Wehmeyer *et al.*, 1996). *Arabidopsis* sequencing project revealed many HSP-related genes (The Arabidopsis Genome Initiative, 2000), and they are classified into five groups, low molecular weight HSP, chaperonin, HSP70, HSP90, and HSP100 family. Chaperonin, HSP70, HSP90 and HSP100 were homologous to GroE, DnaK, HtpG, and ClpB in *E. coli*, respectively. In the *N. glutinosa* leaves, 13 HSP-related genes except HSP100 family were identified as up-regulated genes upon wounding. As described above, the genes encoding some HSPs are induced twice in response to wounding, and therefore, they may play essential roles in defense reaction in response to wounding.

Of 86 genes identified in this study, 15 genes including 11 hypothetical protein genes

and one unknown protein gene were identified as wound-inducible genes. A further study on the wound-inducible genes identified in the present study and/or characterization of their translational products will provide valuable insight into the defense mechanism of plants.

As described above, *N. glutinosa* displays TMV-resistance mediated by *N*-gene at 25 °C, while TMV-infection at 35 °C allows TMV to spread systemically and develop a characteristic mosaic phenotype that is visible approximately 10 days after infection. The present study demonstrated the dependability of the full-length cDNA microarray for a comprehensive analysis of extensive changes of the gene expression. Hence, the microarray analysis of the TMV-inducible genes in the *N. glutinosa* leaves at 25 °C and 35 °C would provide valuable information about the *N*-gene mediated disease resistance.

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