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Research Article

Transcriptional profiles of Rhizobium vitis-inoculated and salicylic acid-treated 'Tamnara' grapevines based on microarray analysis

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Abstract The transcriptional profiles of 'Tamnara' grapevine (*Vitis labruscana* L.) to *Rhizobium vitis* were determined using 12,000 gene oligonucleotide microarray chips constructed with 6,776 unigenes based on the EST sequencing. Among them, 95 clones were up-regulated more than three times and 90 were down-regulated more than 5-times in the *R. vitis*-inoculated grapevines relative to the control vines. Treatment of salicylic acid showed that 337 clones were upregulated and 52 clones were down regulated in grapevines. Microarray analysis, reverse transcription-polymer chain reaction, and slot blot hybridization analysis revealed that 5, 14, and 64 clones were up-regulated and 10, 12, and 61 clones were down-regulated in wounded, salicylic acid-treated, and *R. vitis*-inoculated 'Tamnara' grapevine leaves, respectively. The expression patterns of β-1,3-glucanase, proline-rich protein, and lipoxygenase genes of 'Tamnara' moderately resistant to *R. vitis* were similar to those of resistant 'Concord' and 'Delaware' grapevines. However, chalcone synthase genes in 'Tamnara' grapevines showed similar expression patterns to susceptible grapevines 'Neomuscat' and 'Rizamat'. Further expression studies with various clones for each gene should be conducted to elucidate their roles in resistant responses against pathogens or other stimuli in grapevines. These results could provide better resources for understanding the mechanism of defense responses against crown gall disease and clues for identifying new genes that may play a role in defense against *R. vitis* in grapevines.

Keywords Grape, Gene expression, RT-PCR, Slot blot

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Introduction

Grape (*Vitis* sp.) is attacted to a number of bacterial, fungal, and viral diseases like other plants (Pearson and Goheen 1998). Crown gall is a major disease responsible for severe reduction of yield and poor quality of fruit in grape production regions throughout the world, including Korea (Burr et al. 1998; Park et al. 2000). Chemical or biological attempts to control crown gall disease conducted to date have failed, with the exception of *Agrobacterium radiobacter* strain K84 of the biological control (Anand et al. 2008; Burr and Otten 1999). However, *A. radiobacter* strain K84 is only effective at controlling crown gall caused by nopaline-type strains of *A. tumefaciens* and *A. rhizogenes*, while it has no effect on crown galls induced by *Rhizobium vitis* in grapevines (Kerr 1980). Since effective agro-chemicals to control crown gall have not been released, development of novel grapevine cultivars resistant to crown gall is critical and will be a useful tool in protection of grapevines from disease (Burr et al. 1988; Park et al. 2000; Stover et al. 1997).

There has been continuous study of genes related to disease resistance and defense response of grapevines to fungal, bacterial, and viral pathogens, including crown gall disease through comparative genomics, transcriptomics and the genome wide identification analysis for useful genes and molecular markers (Burr et al. 1998 and 2003; Choi et al. 2008; Hur et al. 2015). However, development of disease resistant grapes based on molecular biology has been limited because of the relatively low amount of genetic and molecular information available regarding genotypes resistant to certain diseases. Although the entire genome of *Vitis vinifera* 'Pinot Noir' has been sequenced (The France-Italian Public Consortium for Grapevine 2007) and annotated, the functions of many genes must be still investigated.

To develop new grape cultivars resistant to diseases, systematic monitoring of the mechanism of plant response and defense against pathogen attacks and more detailed functional studies of the selected differentially expressed genes (DEGs) are required. Microarray analysis, which can screen the expression patterns of many genes simultaneously in a single analysis, is considered a foundational technology capable of high-throughput and high-speed transcriptional profiling. Accordingly, this technique has various applications including unique gene identification and diagnostics of certain diseases (Schulze and Downward 2001; Stears 2003).

In this study, the gene expression patterns in response to *R. vitis* inoculation were investigated in *R. vitis*-inoculated, salicylic acid (SA)-treated, wounded, and untreated control leaves of 'Tamnara' grapevine which was bred in Korea (Park et al. 2004). Using microarray gene expression profiling, 6,776 unigenes of expressed sequence tag (EST)-based sequence were analyzed in grapevine leaves. The detailed expression patterns of selected up- and down-regulated genes in the microarray were confirmed by slot blot hybridization and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). Expression of DEGs was also analyzed in grapevines resistant and susceptible to crown gall in response to *R. vitis* inoculation.

Materials and Methods

Plant materials and treatments

Grapevines of 'Tamnara' (moderately resistant to crown gall disease), 'Delaware' and 'Concord' (resistant to crown gall disease), and 'Neomuscat' and 'Rizamat' (susceptible to crown gall disease) were grown in a greenhouse at 25°C ~ 30°C under natural light, then inoculated with *R. vitis* Cheonan 493 (Yun et al. 2003). Leaves were harvested at 1, 3, 6, 12, 24, 48, and 72 h after wound, SA treatment, and *R. vitis* inoculation, immediately frozen in liquid nitrogen, and stored at -80°C until used for RNA extraction. All samples harvested from each treatment were used for RNA extraction, analysis of differential expression of cDNA, and RT-PCR analysis.

Microarray chip construction

A total of 6,776 unigenes were obtained from the 'Tamnara' grapevine cDNA library constructed after *R. vitis* inoculation and SA treatment. Microarray chips were constructed with 35 –40 nt of unigene based oligonucleotide. Gene specific oligonucleotides were arrayed onto a slide glass with an average of two replications and oligonucleotide microarray techniques were employed to detect *R. vitis*-responsive genes in 'Tamnara' grapevines.

RNA isolation and microarray hybridization

Total RNA was isolated from *R. vitis*-inoculated, SA-treated, and control grapevine leaves using the modified pine tree method of removing polysaccharides and phenolic compounds (Chang et al. 1993) with RNA extraction buffer consisting of 2% cetyltrimethylammonium bromide, 2% polyvinylpyrrolidone, 100 mM Tris-HCl (pH 8.0), 25 mM ethylenediaminetetraacetic acid, 2 M NaCl, 0.5 $g \cdot L^{-1}$ spermidine, and 2% β-mercaptoethanol. To determine the SA and *R. vitis*-responsive genes, the oligonucleotide microarrays were hybridized with probes prepared from the total RNA of SA-treated, *R. vitis*-inoculated, and control leaves.

The MessageAmp™ II-Biotin Enhanced Single Round aRNA Amplification Kit (Ambion, Woodward Austin, TX, USA) is based on the RNA amplification protocol developed in the laboratory of James Eberwine (Van Gelder et al. 1990). Microarray hybridization was performed with 5 μg of a labeled target sample per one CustomArray™ using a 12K microarray hybridized and scanned PMT 500-700, pixel size of 5, focus position 130. Analyses were conducted using a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA, USA). After data extraction, backgrounds for individual samples were calculated. One-way analysis of variance (ANOVA) and a t-test were applied to determine differentially expressed sets of genes across three experimental groups. Statistical significances were adjusted by Benjamini-Hochberg FDR multiple-testing correction (Benjamini and Hochberg 1995). Complete linkage hierarchical clustering based on the Euclidean distances of samples was performed using the normalized significant genes. The patterns of expressed changes were analyzed for groups using the Avadis Prophetic Ver. 3.3 software (StrandGenomics, Bangalore, India, http://avadis. Strandgenomics.com/).

Semiquantitative RT-PCR analysis

Semiquantitative RT-PCR analysis was conducted using 95 up-regulated genes and 90 down-regulated genes. cDNAs was synthesized using a ReverTra-plus-™ High Fidelity RT-PCR Kit (Toyobo, PCR-501, Japan). PCR amplification was conducted by subjecting the samples to 94°C for 2 min, followed by 30 cycles of 98°C for 10 s, 58°C for 30 s and 68°C for 1 min using KOD-Plus taq polymerase (Toyobo, KOD-201, Japan). PCR amplification was conducted using primers

specific for each gene and actin primers as an internal control under appropriate conditions.

RNA slot blot hybridization analysis

Total RNA (5 μg) isolated from the leaves of grapevines was used for the RNA slot blot hybridization analysis. The RNA mixtures were denatured at 65°C for 10 min, then blotted onto membranes using the Bio-Dot SF (BioRad). RNA samples were transferred and immobilized to Hybond-N^+ nylon membrane with UV-crosslinker. Hybridization, washing, detection, and exposure on X-ray film were performed as previously described.

Results and Discussion

Hierarchical clustering of the ESTs derived from 'Tamnara' grapevines

To obtain molecular profiles of responses to *R. vitis* in grapevines, the differential transcriptional profiles of *R. vitis*-inoculated and SA-treated grapevines were investigated using microarray analysis with 12,000 gene oligonucleotides. Samples for each treatment were harvested at multiple time points to screen for a large number of biochemical changes expected to occur after *R. vitis*-inoculation and SA-treatment. Genes showing induced and suppressed expression patterns in grapevines inoculated with *R. vitis* or treated with SA compared to untreated controls were selected as up- and down-regulated and used to validate expression analysis (Fig. 1).

As shown in the Venn diagram, 337 and 27 genes were induced more than two times by SA treatment and *R. vitis* inoculation, respectively (Fig. 1A). Among ten genes commonly up-regulated under both treatments, several were well-known defense-related genes, including proline-rich protein 1, leuco-anthocyanidin dioxgenase, cytosolic heat shock 70

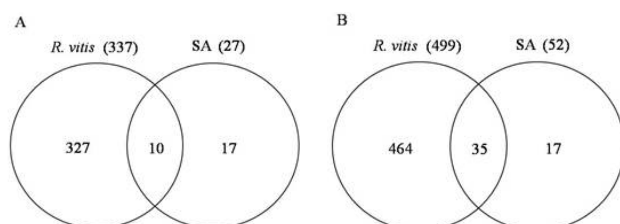

Fig. 1 Venn diagrams showing the numbers of overlapped and unique genes induced (A) and suppressed (B) more than twice in the level of their expressions by *R. vitis* inoculation and salicylic acid (SA) treatment. Results were based on the mean inductions of three replicates

protein, and sarcosine oxidase family protein, while the functions of others were unknown. Similarly, among the ESTs of 'Tamnara' grapevines, 499 genes from *R. vitis*-inoculated and 52 genes from SA-treated samples were down-regulated by more than two times, respectively (Fig. 1B). Additionally, 35 genes were suppressed by both treatments, which include genes encoding heavy metal-associated domain-containing protein, Zn (C3HC4-type RING) finger family protein, putative WRKY transcription factors 4 and 30, MAP kinase-like protein, and hypothetical proteins. It was reported that several transcripts such as subtilisin-like protease, phenylalanine ammonia lyase (PAL), S-adenosylmethionine synthase, WD-repeat protein like, and J2P, were up-regulated in 'Regent' grapevine against the mildew (Figueiredo et al. 2013). Camparative analysis between resistant and susceptible grape cultivars to Pierce's disease showed that significant differences in transcripts including some of the PR proteins such as β-1,3-glucanase and chitinases and proline-rich proteins were shown at the stem tissues infected with *Xylella fastidiosa* (Lin et al. 2007). In this study, it was shown that expressions of various defense-related genes were differentially regulated in the grapevines by *R. vitis* infection and SA-treatment.

R. vitis and SA responsive DEGs with microarray analysis

Overall, 95 up-regulated cDNA clones showed expression that was up-regulate/d by more than 3-times, while 90 down-regulated clones showed decreases in expression of more than 5-times in *R. vitis*-inoculated grapevines relative to untreated samples (Tables 1 and 2). *Arabidopsis thaliana* seed imbibition 2 (ATSI2) hydrolyzing O-glycosyl compound gene and btb and taz domain protein related genes were highly up-regulated. Conversely, cell wall protein, expansin, and endosperm specific protein genes were significantly suppressed. Additionally, the transcriptional levels of defense, signal transduction, active oxygen related genes such as methallothionine-like protein, LRR domains, and PR protein, as well as transcription factors such as MYB transcription factors were highly activated. These results were consistent with those of ESTs from *R. vitis* inoculated 'Tamnara' grapevines (Choi et al. 2010).

As shown in the Tables 1 and 2, some genes showed antagonistic expression patterns between induced genes of *R. vitis*-inoculated and SA-treated grapevines. The genes activated by *R. vitis* inoculation might be mediated by jasmonic acid (JA) or ethylene. The expression of 95 up-regulated genes was suppressed by *R. vitis* inoculation in SA-treated grapevine leaves. In contrast, the expression of 90 genes down-regulated by *R. vitis* inoculation increased in response

Table 1 Genes in 'Tamnara' grapevines up-regulated in response to *R. vitis* inoculation and SA treatment

Gene No. in EPP163JWAA series	No. of slot blot, RT-PCR		Putative function	Ratio of signal intensity	
				R. vitis	SA
12C000256	B9	U1	ATSI 2 hydrolyzing O-glycosyl compounds yabby15 protein	15.9±0.49	-14.2±0.44
12S013353	F4	U2	Btb and taz domain protein	11.2±0.85	-4.2±0.27
12C000750	F2	U3	Metallothionein-like protein	10.3±0.63	-6.0±0.79
12C000845	D7	U4	LOX	9.3±0.52	-11.1±0.66
12C001644	B6	U5	Dark inducible 10 hydrolyzing O-glycosyl compounds	7.6±0.46	-7.8±0.36
12S000483	H3	U6	No hit	7.6±0.70	-3.5±0.81
12S008848	H1	U7	Hypothetical protein	7.4±0.19	-3.1±0.71
12S003419	A7	U8	Glycosyl hydrolase family 1 protein	7.0±0.15	-4.8±0.25
12S007850	G6	U9	CHS	6.8±0.94	-4.5±0.72
12S003388	A3	U10	Glycosyl hydrolase family 1 protein	6.5±0.07	-4.7±0.21
12S009383	F5	U11	Aspartyl protease family protein	6.4±0.57	-4.2±0.63
12S005565	B2	U12	Glucose-6-phosphate translocator	6.3±0.18	-5.5±0.31
12S002570	B10	U13	Limonoid udp-lucosyltransferase	6.2±0.26	-3.6±0.39
12S002915	B3	U14	Cytochrome P450	6.2±0.17	-4.2±0.32
12S009437	A12	U15	ATSI 2 hydrolyzing O-glycosyl compounds	6.0±0.36	-7.3±0.16
12S006649	C4	U16	Isoamylase isoform 3	6.0±0.12	-5.2±0.44
12S000876	D2	U17	Cytochrome p450	6.0±0.43	-4.9±0.33
12S010715	G3	U18	Cytochrome p450	5.9±0.69	-3.4±0.58
12S012744	E11	U19	Proline-rich cell wall protein	5.9±0.49	-2.9±0.53
12S005981	A5	U20	Organic cation transporter	5.8±0.19	-5.0±0.22
12S002410	D4	U21	Thaumatin-like protein	5.8±0.60	-6.9±0.57
12S009693	D11	U22	Starch phosphorylase	5.8±0.44	-4.2±0.34
12C000042	G2	U23	Myb transcription factor	5.7±0.54	-4.1±0.70
12S013702	A6	U24	Aldehyde dehydrogenase	5.7±0.16	-6.0±0.27
12C001360	C1	U25	Protein	5.6±0.33	-6.0±0.38
12S011414	E4	U26	GST	5.6±0.11	-3.9±0.56
12S000490	A2	U27	Mate efflux family expressed	5.5±0.15	-3.5±0.10
12S012305	B12	U28	Hypothetical protein	5.5±0.25	-4.2±0.37
12S006861	C5	U29	Reductase 1	5.3±0.25	-2.7±0.30
12S006594	A11	U30	Molybdenum cofactor sulfurase	4.9±0.22	-6.2±0.28
12S007535	A10	U31	Glyoxalase i	4.6±0.13	-2.3±0.22
12S005609	D8	U32	Expansin-like protein	4.6±0.14	-3.7±0.46
12C000943	D1	U33	Ef-1 a	4.5±0.40	-2.7±0.26
12S011005	C3	U34	Alkaline a galactosidase	4.4±0.35	-4.7±0.33
12S000980	D10	U35	Fatty acid hydroperoxide lyase	4.4±0.29	-3.6±0.39
12S002559	B7	U36	Af303396_1udp-glucosyltransferase hra25	4.4±0.28	-2.9±0.16
12S010472	B11	U37	Tpa:gid1-like gibberellin receptor	4.4±0.33	-3.0±0.06
12S006784	F8	U38	Glucose acyltransferase	4.3±0.50	-3.7±0.35
12S011826	F12	U39	Ethephon-induced protein	4.3±0.49	-3.1±0.50
12S005921	C7	U40	Fibrillin	4.3±0.25	-2.9±0.28
12S009053	E6	U41	Condensation domain-containing protein	4.2±0.46	-3.0±0.11
12C000227	E3	U42	Basic helix-loop-helixfamily protein	4.2±0.14	-2.7±0.44
12C000359	E1	U43	Sucrose synthase	4.1±0.21	-3.6±0.22
12S010980	A9	U44	Pyrroline-5-carboxylate synthetase	4.1±0.44	-5.7±0.34
12S002233	E12	U45	Glyceraldehyde-3-phosphate dehydrogenase	4.1±0.48	-3.5±0.28
12C001111	G5	U46	Chalcone-flavanone isomerase family expressed	4.1±0.63	-2.9±0.54
12S011777	H10	U47	Abscisic acid responsive elements-binding factor	4.0±0.92	-2.3±0.15
12S009704	A4	U48	Hypothetical protein	4.0±0.14	-3.0±0.16
12C001087	H11	U49	Methionine gamma-lyase	3.9±0.30	-2.6±0.92
12S002496	H2	U50	Protein kinase	3.8±0.33	-3.0±0.60

Table 2 Genes in 'Tamnara' grapevines down-regulated in response to *R. vitis* inoculation and SA treatment

Gene No. in EPP163JWAA Series	No. of slot blot, RT-PCR		Putative function	Ratio of signal intensity	
				R. vitis	SA
12C001593	D8	D1	Cell wall protein	-50.2±0.87	36.1±0.56
12S000426	A2	D2	Expansin	-45.6±0.20	32.6±0.32
12S005616	A10	D3	At5g25460 f18g18_200	-33.9±0.47	32.6±0.33
12C000040	F5	D4	Cytochrome C oxidase polypeptide vc	-21.7±0.94	16.0±0.78
12C000966	E7	D5	Endosperm specific	-21.7±0.82	16.1±0.65
12C000783	B1	D6	Protease inhibitor seed storage lipid transfer protein	-21.6±0.40	15.2±0.41
12C000998	E4	D7	Protein	-18.1±0.83	17.4±0.59
12S003758	F1	D8	En/Spm-like transposon protein	-17.9±0.92	15.1±0.48
12C001094	F12	D9	Meiosis 5	-17.8±1.09	13.8±0.89
12S013145	A9	D10	Proline-rich protein apg isolog	-16.4±0.20	11.3±0.37
12S010548	B12	D11	DNA heat shock N-terminal domain-containing protein	-15.9±0.39	12.3±0.49
12C000058	G6	D12	Acid phosphatase	-15.4±1.42	9.4±0.57
12C000034	A6	D13	Expansin	-14.9±0.27	17.3±0.19
12C001244	B6	D14	Heavy metal-associated domain-containing protein	-14.7±0.31	5.5±0.40
12C000247	A5	D15	SA-induced fragment 1 protein	-13.8±0.21	13.4±0.27
12S011527	F2	D16	Cold induced	-13.6±0.84	5.1±0.17
12C000203	A4	D17	Rho GDP-dissociation inhibitor 1	-13.4±0.26	7.8±0.20
12C001631	E2	D18	Proline rich protein 2	-13.1±0.77	11.1±0.26
12S009428	B10	D19	Hypothetical protein	-13.1±0.48	12.5±0.15
12C000933	A7	D20	Fatty acid elongase	-12.8±0.30	9.7±0.21
12S005808	A1	D21	Proline-rich protein	-10.8±0.06	14.1±0.20
12C000939	D5	D22	β-Ketoacyl-synthase	-10.7±0.25	4.5±0.52
12C001125	D10	D23	Xyloglucan endotransglycosylase	-10.6±0.64	10.8±0.43
12S011610	D9	D24	Hypothetical protein	-10.6±0.23	7.8±0.56
12S008346	G7	D25	No hit	-10.0±1.35	12.0±0.57
12S008268	F11	D26	Tonoplast membrane integral protein4-4	-9.9±0.76	5.2±0.48
12S011177	D3	D27	Hypothetical protein	-9.7±0.49	7.5±0.19
12C000816	C6	D28	Chloroplast chlorophyll a/b binding protein	-9.7±0.48	14.7±0.22
12C000797	A8	D29	Chloroplast chlorophyll a/b binding protein	-9.6±0.30	10.5±0.34
12S005860	E5	D30	CCHC-type integrase	-9.5±0.28	3.6±0.65
12C000927	G1	D31	Protein binding protein	-8.8±0.74	5.8±0.51
12S008817	C3	D32	LRR protein	-8.5±0.15	6.2±0.39
12C001369	D4	D33	Transferase family protein	-8.4±0.24	10.3±0.52
12S001577	C1	D34	No hit	-8.4±0.18	3.1±0.34
12S003449	A3	D35	Mee60 (maternal effect embryo arrest 60)	-8.3±0.21	11.9±0.21
12C001193	B9	D36	Yabby15 protein	-8.1±0.28	6.8±0.36
12S009302	G2	D37	Aspartyl protease family protein	-8.0±0.74	5.7±0.73
12S011056	B11	D38	At3g15630 msj11_3	-7.9±0.25	3.0±0.25
12C001299	E1	D39	Endo-β-glucanase precursor	-7.8±0.46	4.7±0.46
12S013533	H2	D40	Expansin-like protein a	-7.7±0.42	2.2±0.66
12S013059	G5	D41	Thaumatin-like protein	-7.7±1.01	7.0±0.62
12C000786	F7	D42	Tonoplast intrinsic protein	-7.6±0.63	5.6±0.49
12S006978	C10	D43	WRKY transcription factor 10	-7.5±0.39	5.3±0.32
12C000038	B8	D44	Hypothetical protein	-7.5±0.32	4.8±0.18
12C001095	G10	D45	α Tubulin 1	-7.3±1.16	3.9±0.58
12S010793	F10	D46	At5g44130 mln1_5	-7.2±0.60	5.8±0.62
12C000621	E8	D47	Chitinase-like protein	-7.2±0.58	5.5±0.29
12C000406	G3	D48	Glutamine synthetase	-7.2±0.76	4.9±0.66
12C000508	B7	D49	Nucleoid DNA-binding protein cnd41	-7.1±0.18	6.7±0.35
12S009795	C5	D50	Calmodulin-like protein	-7.0±0.29	5.2±0.35

Table 3 Defense-related cDNA responsive to *R. vitis* inoculation and SA treatment in ‘Tamnara’ grapevines

Gene	Putative function/homology	Ratio of signal intensity	
		R. vitis	SA
Defense-related	β-1,3-Glucanase	-3.4±0.36	-1.4±0.38
	Chitinase	-4.4±0.25	-1.2±0.26
	Basic endochitinase precursor	2.3±0.37	-1.7±0.32
	Chitinase III	-2.6±0.51	-1.4±0.66
	Thaumatin-like protein	5.8±0.60	-1.3±0.24
	Chalcone synthase	6.8±0.94	1.5±1.11
Signal transduction	LOX	9.3±0.52	-1.2±0.69
	NBS LRR-containing protein	3.5±0.73	1.2±0.81
Active oxygen related	Catalase	2.5±0.71	-1.1±0.70
	Glutathione peroxidase	3.2±0.19	1.1±0.42
	Glutathione S-transferase	5.6±0.11	1.4±0.56
	Ascorbate peroxidase	1.8±0.25	1.1±0.23
Secondary metabolites	Cytochrome P450	6.0±0.43	-4.9±0.33
	Cytochrome C oxidase subunit Vb	1.8±0.21	1.1±0.33
Abiotic stress-related	Small heat shock protein	3.5±0.29	1.3±0.35
	Heat shock protein	3.7±0.18	1.6±0.32
Cell wall fortification	Proline-rich cell wall protein	5.9±0.49	2.0±0.43
Transcription factors	WRKY transcription factor	1.7±0.21	-1.1±0.26
	MYB transcription factor	5.8±0.54	1.4±0.84

Fig. 2 Semiquantitative RT-PCR analysis of 95 highly up-regulated genes in ‘Tamnara’ grapevines. cDNA samples were amplified with a ReverTra-plus™-High Fidelity RT-PCR Kit. MAU: up-regulated in microarray analysis; C, control; W, wound; S, SA treatment; R, *R. vitis* inoculation; M, size marker

Fig. 3 Semiquantitative RT-PCR analysis of 90 highly down-regulated genes in ‘Tamnara’ grapevines. cDNA samples were amplified with a ReverTra-plus™-High Fidelity RT-PCR Kit. MAD: down-regulated in microarray analysis; C, control; W, wound; S, SA treatment; R, *R. vitis* inoculation; M, size marker

to SA treatment. When *R. vitis* attacks the vines, highly induced JA-dependent responses might suppress the gene expression involved SA-dependent defense pathway due to pathway crosstalk by *R. vitis*. Antagonisms between JA or ethylene and SA signaling have been extensively studied (Dong 1998; Kunkel and Brooks 2002). A large number of genes, includingc lipoxygenase (LOX), lipid transferase, and genes related to secondary metabolisms (Creelman and Mullet 1997; Lin et al. 2007) that were involved in JA biosynthesis, have been shown to respond to wounding and *R. vitis* attack in grapevines. The crosstalk among a number of signaling molecules appears to be related to controlling defense systems in response to pathogen attacks in grapevines as in other plants (Shah 2003).

Genes in response to *R. vitis*, SA, and wound in 'Tamnara' grapevines

Transcriptional profiling in response to *R. vitis* with the 6,776 unigenes obtained from the *R. vitis*-inoculated and SA-treated 'Tamnara' grapevine cDNA library was conducted by use of gene specific oligonucleotide microarray chips. cDNA contigs responsive to *R. vitis* inoculation and SA treatment could be categorized into seven groups encoding proteins involved in defense, defense signaling, oxidative burst, secondary metabolism, abiotic stress, cell wall fortification, and transcription factors (Table 3). The expression level of 95 up-regulated and 90 down-regulated ESTs was confirmed by semiquantitative RT-PCR (Fig. 2 and 3) and RNA slot blot hybridization analysis (Fig. 5 and 6). Confirmation using the three expression profiling methods revealed that 5, 14, and 64 cDNAs were up-regulated by wound, SA-treatment, and *R. vitis* inoculation, while 10, 12, and 61 were down-regulated by each treatment, respectively (Table 4).

As shown in the Venn diagram (Fig. 4A), 50 cDNAs, including ATSI protein 2 hydrolyzing O-glycosyl compounds yabby15 protein, CHS, cytochrome P450, thaumatin-like protein, GST, and LRR containing protein, were specifically activated by *R. vitis* inoculation in 'Tamnara' grapevine leaves. However, no cDNA was shown to be up-regulated by wound or SA treatment in grapevines. Five genes, LOX, aspartyl protease family protein, heavy metal-associated domain-containing protein, tyrosine aminotransferase, and one with unknown function, were commonly induced by *R. vitis* inoculation, wound, and SA treatments. Similarly, 1, 4, and 48 cDNAs were down-regulated by wound, SA treatment and *R. vitis* inoculation, respectively (Fig. 4B). Expansin, cytochrome C oxidase polypeptide vc, seed storage lipid transfer protein, meiosis 5, SA-induced fragment 1 protein, WRKY transcription factor 10, chitinase-like protein, and Zn finger (gata type) family proteins were specifically down-regulated by *R. vitis* inoculation. Four genes such as lipid transfer protein, extensin-like protein, and two proteins with unknown function were commonly inhibited under both *R. vitis* inoculation and SA treatment (Table 5). More detailed functional studies should be conducted to determine if they are involved in defense mechanisms. It has been reported that PR genes were activated by endo- and exogenous SA treatments and pathogen attacks in many plants (Malamy 1990). In this study, genes involved in plant defense responses such as thaumatin-like protein, chalcone synthase (CHS), and LOX were induced by *R. vitis* inoculation and SA treatment.

In this study, some genes such as β-1,3-glucanase and chitinase III responded similarly to *R. vitis* inoculation and SA treatment. Using the GeneFishing and RACE technology, the full-length cDNA of several PR genes including β-1,3 glucanase, PR 10, and thaumatin-like proteins expressed specifically by *R. vitis* inoculation and SA treatment were cloned from the grapevine leaves (Choi et al. 2008). Cheong et al. (2002) reported that various genes such as LOX, catalase

Table 4 Genes specifically expressed in response to wound, SA treatment, and R. vitis inoculation with microarray, RT-PCR, and slot blot hybridization analyses in 'Tamnara' grapevines

A. Up-regulated genes

Confirming method	Wound	SA	*R. vitis*
Microarray and slot blot	78	37	82
Microarray and RT-PCR	7	32	73
Microarray, slotblot, and RT-PCR	5	14	64

B. Down-regulated genes

Confirming method	Wound	SA	*R. vitis*
Microarray and slot blot	70	64	71
Microarray and RT-PCR	14	16	74
Microarray, slotblot, and RT-PCR	10	12	61

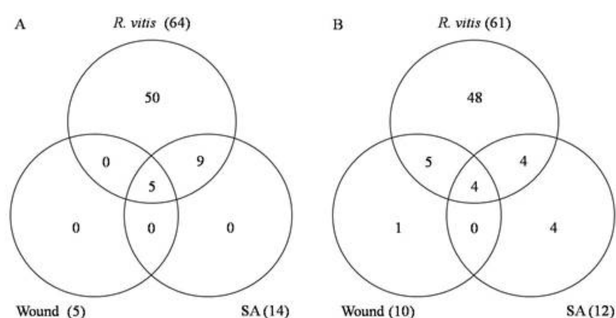

Fig. 4 Venn diagram of DEGs upregulated (A) and down-regulated (B) in 'Tamnara' grapevines responsive to *R. vitis* inoculation, wound, and SA treatment

(CAT), glutathione *S*-transferase (GST), cytochrome P450, and WRKY, as well as MYB transcription factors were activated by wound stress in grapevines. A number of components in their signaling pathways appear to share the response against pathogen infection and wounding stress (Cheong et al. 2002; Maleck and Dietrich 1999). Accordingly, further functional analysis is required to determine the expression dynamics of selected genes in response to mechanical wounding, *R. vitis* inoculation, and SA treatment.

Semiquantitative RT-PCR and slot blot hybridization analysis of cDNA

The expression level of 95 up-regulated and 90 down-regulated ESTs was confirmed by semiquantitative RT-PCR (Fig. 2 and 3) and RNA slot blot hybridization analysis (Fig. 5 and 6). Confirmation using the three expression profiling methods revealed that 5, 14, and 64 cDNAs were up-regulated by wound, SA treatment, and *R. vitis* inoculation, while 10,

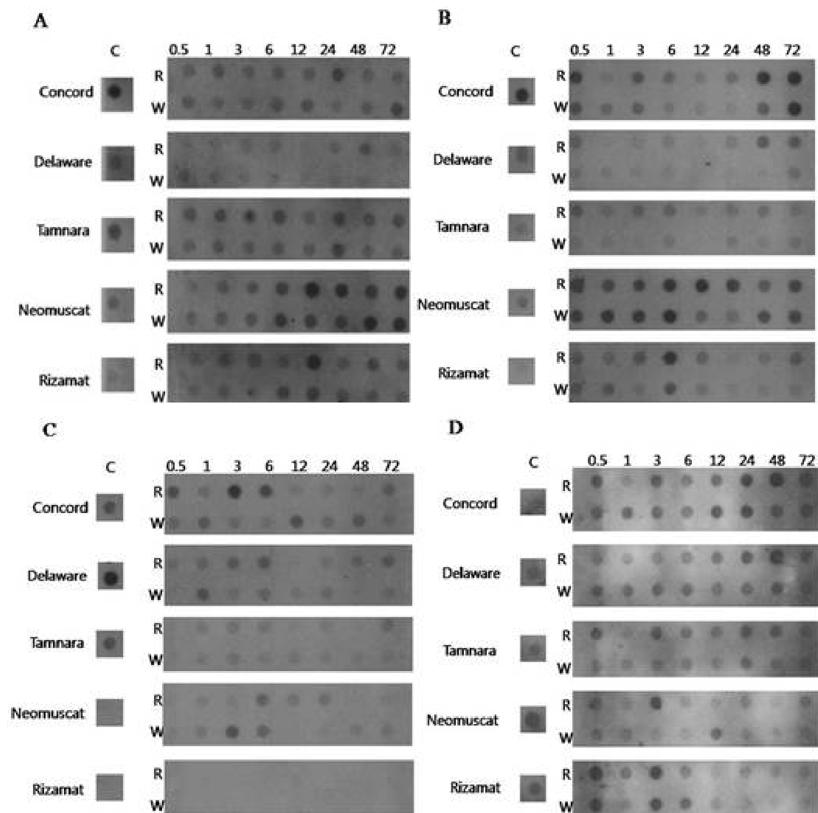

Fig. 5 RNA slot blot hybridization analysis with (A) β-1,3-glucanase, (B) CHS, (C) LOX, and (D) proline-rich protein as a probe in several grapevine cultivars. C, control; R, *R. vitis* inoculation; W, wound

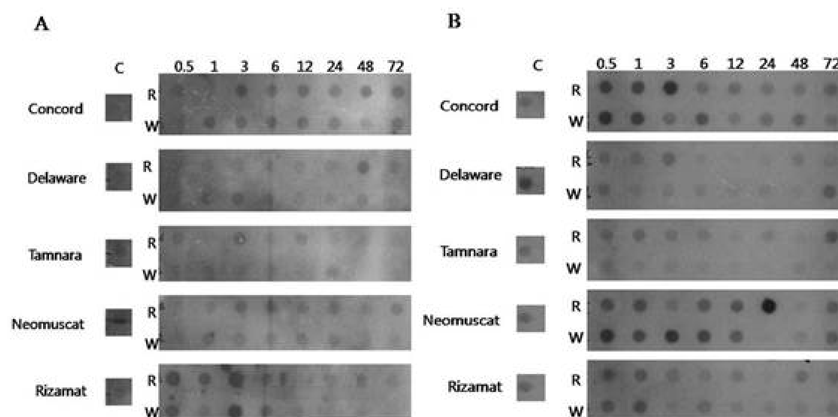

Fig. 6 RNA slot blot hybridization analysis with (A) ATSI 2 and (B) organic cation transporter as a probe in several grapevine cultivars. C, control; R, *R. vitis* inoculation; W, wound

Table 5 DEGs responsive to *R. vitis* inoculation, wound, and SA treatment in 'Tamnara' grapevines

	Up-regulated	Down-regulated
R. vitis	ATSI protein 2 hydrolyzing O-glycosyl compounds yabby15 protein CHS Cytochrome P450 Thaumatin-like protein GST Sucrose synthase Small heat shock protein LRR containing protein	Expansin Cytochrome C oxidase polypeptide vc Seed storage lipid transfer protein Meiosis 5 Proline-rich protein apg isolog SA-induced fragment 1 protein WRKY transcription factor 10 Chitinase-like protein Zn finger (gata type) family protein
Wound	-	Fasciclin-like arabinogalactan protein 14
SA	-	Chloroplast chlorophyll a/b binding protein β-Glucanase-like protein Pollen-specific protein
R. vitis and SA	Btb and taz domain protein Limonoid UDP-glucosyltransferase Alkaline α galactosidase	Tonoplast membrane integral protein4-4 Histone h3
R. vitis and Wound	-	Cell wall protein Glucose-methanol-cholineoxidoreductase family protein
R. vitis, SA, and Wound	LOX Aspartyl protease family protein Heavy metal-associated domain-containing protein Tyrosine aminotransferase	Lipid transfer protein Extensin-like protein

12, and 61 were down-regulated by these treatments, respectively (Table 4).

As shown in the Venn diagram (Fig. 4A), 50 cDNA samples, including ATSI protein 2 hydrolyzing O-glycosyl compounds yabby15 protein, CHS, cytochrome P450, thaumatin-like protein, GST, and LRR containing protein, were specifically activated by *R. vitis* inoculation in 'Tamnara' grapevine leaves. However, no cDNA was shown to be up-regulated by wound or SA treatment in grapevines. Five genes, LOX, aspartyl protease family protein, heavy metal-associated domain-containing protein, tyrosine aminotransferase, and one with unknown function, were commonly induced by *R. vitis* inoculation, wound, and SA treatments. Similarly, 1, 4, and 48 cDNAs were down-regulated by wound, SA treatment and *R. vitis* inoculation, respectively (Fig. 4B). Expansin, cytochrome C oxidase polypeptide vc, seed storage lipid transfer protein, meiosis 5, SA-induced fragment 1 protein, WRKY transcription factor 10, chitinase-like protein, and Zn finger (gata type) family proteins were specifically down-regulated by *R. vitis* inoculation. Four genes were commonly inhibited under both *R. vitis* inoculation and SA treatment, lipid transfer protein, extensin-like protein, and two proteins with unknown function (Table 5). The function of these genes is not clear yet, accordingly, more detailed functional studies should be conducted to determine if they are involved in defense mechanisms.

Comparative analysis of defense-related gene expression in *R. vitis*-inoculated grapevines

To investigate the expression of selected genes, RNA slot blot hybridization was performed using 'Delaware' and 'Concond', 'Neomuscat' and 'Rizamat', and 'Tamnara' grapevine leaves harvested at several time courses. When β-1,3-glucanase, *CHS*, *LOX*, proline-rich cell wall protein, *ATSI 2*, and organic cation transporter genes were used as probes for the hybridization analyses, differential expression was observed between resistant and susceptible cultivars in response to *R. vitis* inoculation and wound treatment (Fig. 5 and 6). The expression patterns of β-1,3-glucanase, proline-rich protein, and *LOX* genes in 'Tamnara' grapevines were similar to their expressions in 'Concord' and 'Delaware' which are resistant to crown gall disease. Although proline-rich cell wall protein genes were highly expressed within 12 to 72 h in 'Concord', 'Delaware', and 'Tamnara' grapevines inoculated with *R. vitis*, they showed low initial expression level, and increased level from 30 min to 12 h after *R. vitis* inoculation in 'Neomuscat' and 'Rizamat' grapevines, which are susceptible to crown gall. Conversely, the *CHS* gene in 'Tamnara' grapevines was expressed at levels similar to those of 'Neomuscat' and 'Rizamat' grapevines. Although transcripts of *CHS* increased at 48 to 72 h after *R. vitis* inoculation and wound treatment, they showed low expression at 0.5 to 12 h after *R. vitis* inoculation in 'Neomuscat', 'Rizamat', and

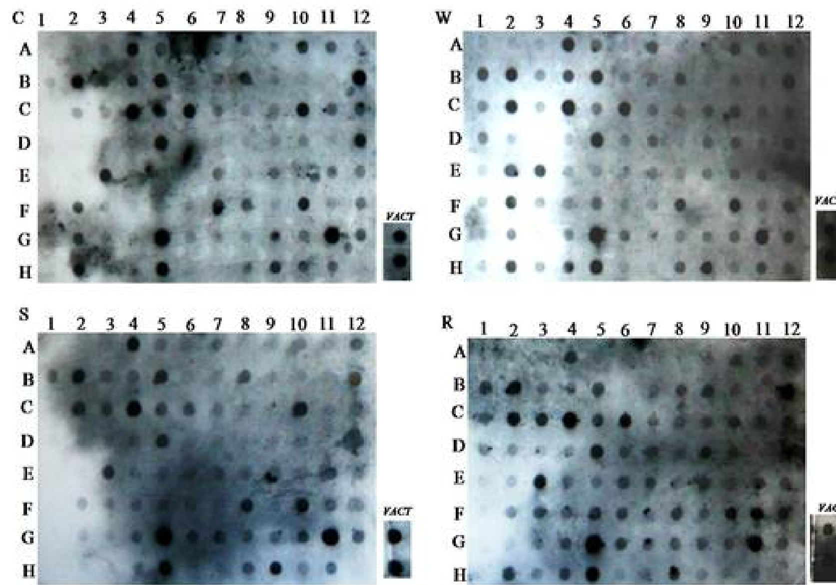

Fig. S1 Slot blot hybridization analysis of 95 highly up-regulated genes in 'Tamnara' grapevines. cDNA probes were synthesized with a ReverTra-plus^{TM}-High Fidelity RT-PCR Kit. C, control; W, wound; S, SA treatment; R, *R. vitis* inoculation; VACT, actin cloned from 'Tamnara' grapevines

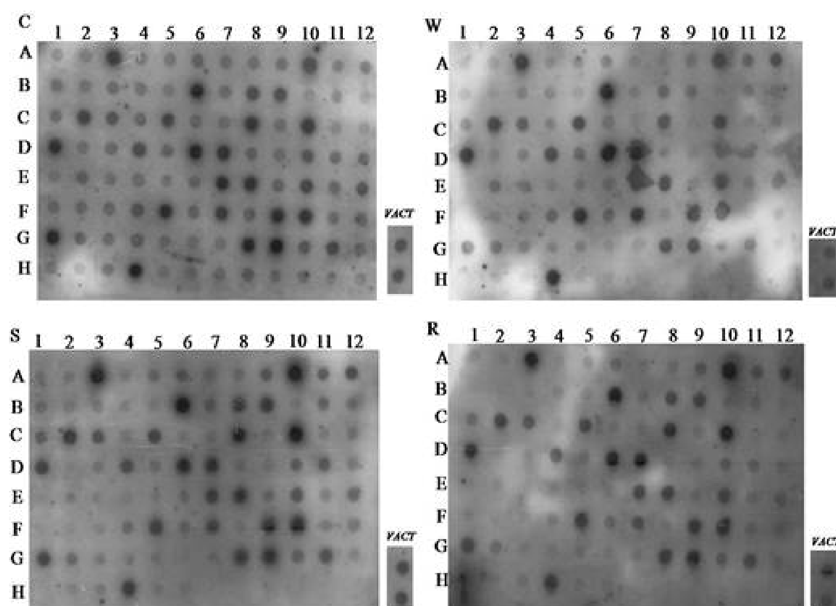

Fig. S2 Slot blot hybridization analysis of 90 highly down-regulated genes in 'Tamnara' grapevines. cDNA probes were synthesized with a ReverTra-plus^{TM}-High Fidelity RT-PCR Kit. C, control; W, wound; S, SA treatment; R, *R. vitis* inoculation; VACT, actin cloned from 'Tamnara' grapevines

'Tamnara' grapevines. In the case of comparative analysis by Lin et al. (2002), PR genes were expressed in both resistant and susceptible grapevine cultivars in response to *X. fastidiosa* inoculation. These findings suggest that susceptible cultivars have a host defense response mechanism that responds to *X. fastidiosa* inoculation, but they may fail to protect themselves from pathogen infections.

ATSI2 and organic cation transporter genes were highly activated by *R. vitis* inoculation in 'Tamnara' grapevines as confirmed by microarray, RT-PCR, and slot blot hybridization analyses (Fig. 6). ATSI 2, formerly raffinose synthase, is a key enzyme that transfers sucrose into the raffinose oligosaccharide, an oligosaccharide commonly found in plant seeds and other tissues (Nishizawa et al. 2008). This gene is known to be activated by abiotic stressors such as frost, drought, and salt (Kandler and Hopf 1984; Keller and Pharr

1996; Peterbauer and Richter 2001; Peterbauer et al. 2000). The results of the present study suggest that expression of the ATSI2 gene was also related to the defense responses to *R. vitis* infection in grapevines.

To understand the resistant responses to disease in grapevines, it is important to monitor the specific expression of genes in response pathogen attacks or signal molecules accumulated by pathogens in vines. Among various strategies to screen for specific gene expression, microarray analysis can be used to analyze variations in the expression of thousands of genes simultaneously (Meyers et al. 2004; Schulze and Downward 2001; Stears 2003). In grapevines, microarray analysis has been used in investigations of transcriptomes related to berry development (Deluc et al. 2007; Terrier et al. 2005; Waters et al. 2006), water and salinity stress (Cramer et al. 2007), ultraviolet-B radiation (Pontin et al. 2010), and virus (Espinoza et al. 2007) and fungal infection (Figueiredo et al. 2008).

In this study, the gene expression patterns in response to *R. vitis* bacterium causing crown gall in grapevines were investigated in *R. vitis*-inoculated, salicylic acid (SA)-treated, wounded, and untreated 'Tamnara' grapevines. Microarray analysis using 12,000 gene oligonucleotides of microarray chips constructed with 6,776 unigenes based on EST sequencing revealed that 95 clones were up-regulated by more than 3 times and 90 were down-regulated by more than 5 times in *R. vitis*-inoculated 'Tamnara' grapevines than in untreated vines. Among these, 5 to 61 up-regulated genes, and 10 to 61 clones showed different expression levels in response to wound, SA, and *R. vitis* in grapevines upon RT-PCR and slot blot hybridization analysis. Some genes, such as β-1,3-glucanase, proline-rich protein, and *LOX*, were induced in moderately resistant cultivars, while others, such as *CHS*, were expressed in moderately resistant and susceptible grapevines in response to *R. vitis*. Further expression studies with various clones per each gene should be conducted to elucidate their roles in resistant responses to pathogens or other stimuli in grapevines. Identification and characterization of the putative genes involved in defense response will be useful for breeding grapes resistant to crown gall. These results could provide a better understanding of the mechanisms of defenses against crown gall disease and clues for identifying new genes that may play a role in defense responses to infection of *R. vitis*.

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