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Global Analysis of Gene Expression upon Acid Treatment in *Arabidopsis thaliana*

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To obtain global gene expression profiles of *Arabidopsis thaliana* by acid stress, seedlings were subjected to low pH stress. Using Affymetrix AH1 chips covering 24,000 genes, we analyzed gene expression patterns. Fifty-four genes were up-regulated, and 38 were down-regulated more than 3-fold after 2 h of acid stress (pH 3.0). Several defense and abiotic stress-related genes were recognized among the up-regulated genes and peroxidase and extensin genes were identified among the down-regulated genes. After 12 h treatment, relatively fewer genes showed changed expression, indicating that plants seem to adjust themselves to this abiotic stress. Most of the up-regulated genes are already known to be involved in abiotic stress responses and pathogen attacks, especially wounding. However, down-regulated genes for the members of extensins and peroxidases are specific to the acid treatment. These results suggest that acid treatment turns on genes involved in stress responses, especially in wounding and turns off genes very specific for the acid stress.

Keywords **:** abiotic stress, acid, *Arabidopsis*, gene expression, microarray

Acid rain is defined as rainfall that has a pH level of less than 5.6. Acid rain deposition primarily results from the oxidation of sulphur dioxide (SO_2) and nitrogen oxides into strong acids such as sulphuric acid (H_2SO_4), and nitric acid (HNO_3). Sulfur dioxide and nitrogen oxides come from the burning of fossil fuels such as coal, natural gas, and oil. Acid rain leaches nutrients from soils, slows the growth of trees, and kills fish and other wildlife. Acid rain can also cause damage to plants both directly and indirectly. It can destroy cuticle layers on leaves, and can cause membrane leakage of ions, resulting in leaf necrosis (Evans and Curry, 1979). Indirectly, acid rain can weaken plants by affecting the availability of nutrients that are essential for plant growth. Additionally, excess hydrogen ions can act upon the soil chemistry, releasing toxic substances such as aluminum, mercury and manganese into the soil, which can inhibit root growth and damage roots (Foy, 1984, 1988; Delhaize and Ryan, 1995).

Arabidopsis is a well-known model plant for plant research, including a few known studies on the effect of acid rain. Park and Lee (1999) observed the different response of Arabidopsis ecotypes to various pH conditions, introduced as simulated acid rain. They found that low pH causes damage to the cuticle layer and necrosis on Arabidopsis leaves, which can cause internal structural changes in leaves (Park and Lee, 1999). Further, leaf necrosis caused by acid rain is similar to necrotic lesions caused by plant pathogens. Lee et al. (2006) discovered that acid rain causes the up-regulation of genes induced by the salicylic acid (SA)-mediated pathogen resistance pathway, however, the expression level of a vegetative storage protein (VSP), one component of the jasmonic acid pathway, was not significantly changed.

With the exception of these studies, not much is known about acid rain effect on Arabidopsis. Specifically, no extensive genetic information is available. Therefore we used Affymetrix AH1 chips covering 24,000 genes to obtain global gene expression profiles of Arabidopsis following acid treatment. Using these tools, we observed extensive changes of gene expressions by acid treatment. These data would provide valuable basic information for deciphering acid-resistant signaling pathways and developing acid-tolerant plants.

Materials and Methods

Plant Materials and acid treatment. Seeds of *Arabidopsis*, ecotype Columbia-0 (Col0) were sterilized for 5 min in 1% sodium hypochlorite, 5 min in 70% EtOH, then washed in sterile water, placed in the dark at 4°C for 2 days, and sown on one-half MS agar plates with 2% sucrose (Murashige and Skoog, 1962). Plants were grown at 23°C, 50-70% relative humidity (RH) in a growth room under a long day (16 h light/8 h dark) light regime. To observe the effect of

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acid on seedling growth, *Arabidopsis* seeds were germinated on sterilized 3 MM gel-blot paper soaked with MS liquid media of pH 5.6, pH 3.0, and pH 2.5, respectively. The pH of MS liquid media was adjusted with 0.1 N H_2SO_4 and seedling growth was observed after 7 days of sowing. For microarray experiments, nine day-old seedlings grown on one-half MS plates were transferred into one-half MS liquid medium of pH 5.6 (control group) and pH 3.0 (experimental group), respectively. To identify as many genes as possible, which can be regulated by direct contact by acid stress or indirectly through signal transduction at different time point, only roots of seedlings were dipped into medium. Control and acid-treated whole seedlings were harvested after 2 and 12 hours of acid treatment and used for total RNA extraction.

Microarray analysis. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The total RNA was quantified using NanoDrop® ND-1000 (NanoDrop, USA) and RNA integrity were determined by Agilent Bioanalyzer 2100 (Agilent Technologies, USA). The GeneChip® Arabidopsis ATH1 Genome Array containing more than 22,500 probe sets representing approximately 24,000 genes was used for microarray experiment. Double-stranded cDNA was synthesized from 5 μg of template total RNA using One-Cycle cDNA Synthesis kit (Affymetrix, USA), and biotin labeled cRNA was synthesized using IVT Labeling kit (Affymetrix, USA). The labeled cRNA was purified with GeneChip® Sample Cleanup Module (Affymetrix, USA). The quality and quantity of the cRNA was checked by conducting gel electrophoresis and NanoDrop® ND-1000 (NanoDrop, USA), respectively. Twenty micrograms of the purified cRNA of each sample was fragmented and hybridized to arrays for 16 h at 45°C. All arrays were washed and stained automatically by using a fluidics Station 450 (Affymetrix, USA) and scanned by GeneChip® scanner 3000 (Affymetrix, Inc, U.S.A). All procedures were performed according to the manufacturer's protocols (Affymetrix, U.S.A). Image processing was performed using Affymetrix GeneChip® Operating System (GCOS). The differentially expressed genes were selected at 2 or 3-fold changes at 2 h and 12 h group after acid treatments compared with the control group. Data were statistically analyzed by Student's t-test and *P*-values <0.01 were considered as statistically significant. The selected genes were filtered by extensin GCOS filtering. All selected genes were analyzed by two-dimensional hierarchical clustering based on Pearson correlation and Complete Linkage. Discrepancies among control, acid treatment group were visualized by Principal component analysis (PCA). For functional classification, Affymetrix ATH1 Chip annotation was used based on data from the TIGR database.

Reverse transcription-polymerase chain reaction (RT-PCR). Extracted RNA was treated with DNase to completely remove residual genomic DNA. First strand cDNA was synthesized from total RNA (3 μg) by reverse transcription using the oligo (dT)-primer as the antisense primer. The first strand reaction was used for subsequent PCR reactions to detect gene expression using gene-specific primers designed from the coding sequence of each gene. RT-PCR was performed for 15 to 40 cycles at 5-cycle intervals, and the best RT-PCR results showing non-saturating levels of amplified gene expression were chosen to quantify the transcript level of each gene (Choi et al., 2004). The RT-PCR of *Ubiquitin* was used as the internal standard. The following primer sets used for RT-PCR are as follows; At2g02990 [forward primer (F): 5'-AATGGGA-GAAGCATGGTA-3', reverse primer (R): 5'-AACCCAA-GGAGTGAAACC-3'], At2g34930 (F: 5'-TCAGAACTG-AGTCGCTTGGA-3', R: 5'-GGAAGTGGTTTCCCACA-GAG-3'), At4g31800 (F: 5'-GCGTTGGAGAAAAACG-

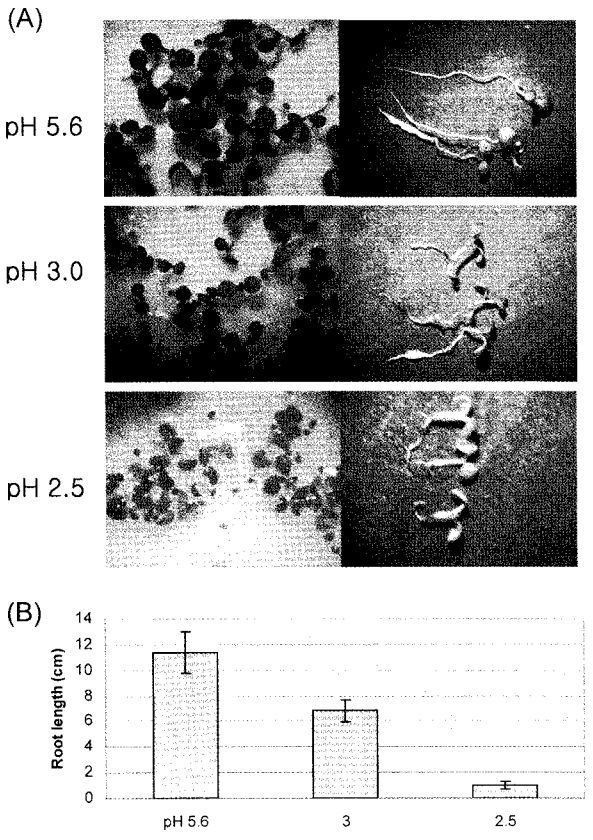


Fig. 1. Effect of acid rain on seedling growth. (A) Arabidopsis seedlings grown on MS medium of different pHs. Photographs were taken 8 days after sowing. (B) Average root lengths of 8-day-old seedlings grown on different pHs. Forty samples were counted for each treatment.

AAAG-3', R: 5'-GACACAATTCGAAAACGAAGAA-3'), At4g37160 (F: 5'-CTTGGTTGATGCTGTTCCAA-3', R: 5'-GAGGAGGCTCAGCTTCAGTG-3'), and *UBQ10* (F: 5'-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3', R: 5'-CGACTTGTCATTAGAAAGAAAGAGATAACA-GG-3').

Results and Discussion

Growth of seedlings was observed on MS plates of different pHs to decide adequate pH for experiments. Seedlings cultured in pH 3.0 and pH 2.5 showed retarded growths of shoots and roots, especially roots were more sensitive to low pH (Fig. 1). Lengths of roots grown in pH 3 and pH 2.5 were reduced to 6.7 cm and 1.1 cm compared to that in pH 5.6 (11.4 cm), respectively (Fig. 1B). In pH 2.5, seedling growth was severely retarded. Shoots became slender, and purple pigment accumulation was visible (Fig. 1A). Purple pigment, usually caused by anthocyanin accumulation in many plants can be induced by biotic and abiotic stresses (Brosché and Strid, 2003). Thus, it indicates that acid imposed stress on *Arabidopsis*, and plants were responding to this stress. Based on acid treatment shown in other publications (Lee et al., 2006; Park and Lee, 1999) and in our data, we decided to treat seedlings with acid of pH 3.

To identify genes regulated at different time point, seedlings were treated at pH 3 for 2 and 12 hours, respectively. Results of cDNA microarray were summarized in Fig. 2. Fifty four genes were up-regulated and 38 were down-regulated more than 3-fold. Of the up-regulated genes, 19 (35%) were related to plant metabolism, 9 (17%) were defense genes and 8 (15%) were abiotic stress-related (Fig. 2A). For those genes repressed, 9 were related to metabolism (24%), and 8 (21%) abiotic stress-related genes were identified. Also 11 (29%) genes encoding cell wall proteins, extensins, were down-regulated (Fig. 2B). For 12 h treatment, relatively fewer genes showed changed expression compared to 2 h treatment. Only 12 genes were up-regulated. All of them belonged into abiotic stress, defense, and metabolism-related genes except 2 unknown genes (Fig. 2C). Nine genes were down-regulated by 12 h acid treatment: 3 unknown genes, 3 abiotic stress, and 3 metabolism-related genes (Fig. 2D). These data indicate that plants somehow adjusted themselves to this abiotic stress after 12 h than 2 h acid treatment. The detailed lists of genes regulated by acid were summarized in Table 1-5. Most of up-regulated genes were those already known to be involved in other abiotic stress responses and pathogen attacks. Cinnamoyl-CoA reductase (CCR2, At1g80820), known to be involved in plant defense and biosynthesis of

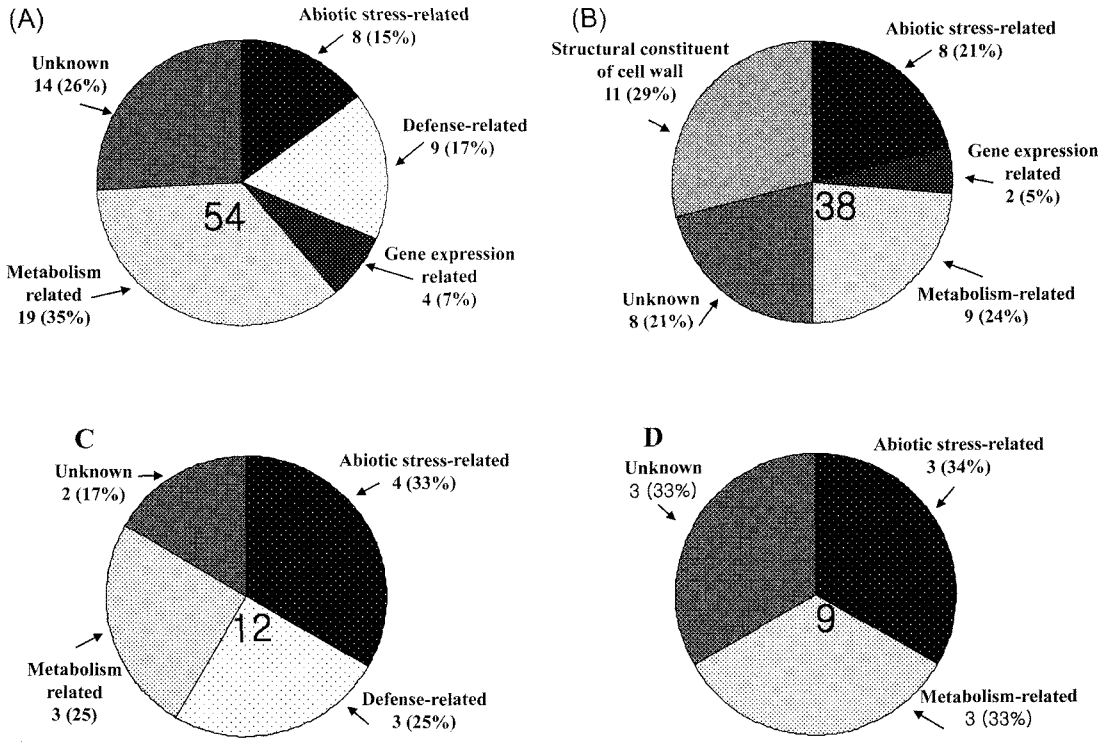


Fig. 2. Functional classification of genes in which expression levels were changed. (A) Up-regulated genes by more than 3-fold upon 2 h acid (pH 3.0) treatment. (B) Down-regulated genes by more than 3-fold upon 2 h acid treatment. (C) Up-regulated genes by more than 3-fold upon 12 h acid treatment. (D) Down-regulated genes by more than 2-fold upon 12 h acid treatment. Numbers indicate total number of genes in which expression level was changed.

Table 1. Up-regulated genes by more than 3-fold upon 2 h acid (pH 3.0) treatment

Gene ID	Fold change		Name
	2h treatment	12h treatment	
Abiotic stress-related			
AT3G23250	12.0	2.7	Unknown (DNA binding)
AT1G80820	7.0	3.7	CCR2 (Cinnamoyl CoA reductase)
AT5G62520	6.1	1.8	SRO5 (Similar to RCD ONE 5)
AT4G10270	4.8	1.7	Unknown
AT1G17420	4.6	2.5	LOX3 (Lipoxygenase 3)
AT2G02990	3.5	7.4	RNS1 (Ribonuclease 1)
AT5G27420	3.5	1.8	Ubiquitin-protein ligase
AT4G11660	3.5	−1.1	AT-HSFB2B (Heat stress transcription factor B-2b)
Defense-related			
AT2G46400	6.0	2.1	WRKY46 (transcription factor)
AT2G43510	5.1	1.3	ATTI1
AT2G34930	4.8	5.8	Unknown (protein binding)
AT1G80840	4.7	1.7	WRKY40
AT4G37990	4.2	3.4	ELI3-2 (Elictor-activated gene 3)
AT2G38470	3.8	2.1	WRKY33
AT3G23240	3.6	−1.1	ATERF1/ERF (Ethylene responsive element)
AT4G31800	3.4	1.2	WRKY18
AT5G49520	3.4	2.6	WRKY48
Gene expression-related			
AT2G47520	8.7	−1.5	Transcription factor
AT3G10040	5.4	3.8	Transcription factor
AT1G64380	4.7	1.5	Transcription factor
AT3G55980	3.5	1.1	Transcription factor
Metabolism-related			
AT3G48520	10.0	2.2	CYP94B3
AT3G50930	8.0	1.7	ATPase
AT4G22610	7.1	2.0	Lipid binding
AT3G27140	6.3	1.1	Protein phosphatase type 2C
AT1G28480	6.2	1.7	Electron transporter
AT1G76650	6.1	2.1	Calcium ion binding
AT4G25810	4.7	2.2	XTR6 (Xyloglucan endotrans glycosylase 6)
AT5G01300	4.2	1.1	Phosphatidylethanolamine binding
AT1G66160	3.8	1.3	Ubiquitin-protein ligase
AT3G62260	3.6	1.6	Protein phosphatase type 2C
AT3G56710	3.6	1.7	SIB1 (Sigma factor binding protein 1)
AT2G48010	3.4	1.4	RKF3 (Receptor like kinase in flowers 3)
AT4G02330	3.4	1.8	Pectinesterase
AT3G59080	3.2	1.5	Pepsin A
AT5G57560	3.2	1.8	TCH4 (TOUCH 4 hydrolase)
AT1G74010	3.0	1.4	Strictosidine synthase
AT5G59550	3.0	1.1	Ubiquitin-protein ligase
AT2G22500	3.0	1.9	Transporter
AT3G25610	3.0	2.0	ATPase
Unknown			
AT1G17380	9.4	3.6	Unknown
AT3G16860	6.1	1.7	Unknown
AT3G02550	5.8	2.0	Unknown
AT5G65300	5.5	1.2	Unknown
AT1G33055	5.3	1.5	Unknown
AT2G27080	5.3	1.5	Unknown
AT1G61340	4.6	1.8	Unknown
AT2G18210	4.5	1.2	Unknown
AT1G20310	4.2	0.8	Unknown
AT3G04640	3.9	1.0	Unknown
AT1G19180	3.5	1.4	Unknown
AT4G24110	3.4	1.3	Unknown
AT3G57450	3.1	1.1	Unknown
AT1G58420	3.0	1.4	Unknown

lignins (Derikvand et al., 2008), accumulated its mRNA seven-fold after 2 h treatment and still showed 3.7-fold increase after 12 h treatment (Table 1). Lipoxygenase (At1g17420), its mRNA accumulates in response to water deficit or wounding (Bell and Mullet, 1991), and pathogen (Melan et al., 1993; Ocampo et al., 1986) also showed increased expression (Table 1). A secreted ribonuclease (At2g02290), another inducible gene by wounding (LeBrasseur et al., 2002) showed increased level of mRNA at 2 h but much higher level-7.4 fold-after 12 h treatment (Table 1). This indicates plants try to adjust to acid stress through signaling pathway that be shared with other abiotic and biotic stresses. Several family members of WRKY also showed increased levels of transcripts (Table 1). WRKY proteins are involved in the defense against pathogens and induced in the responses to the abiotic stresses of wounding, drought and heat, and cold (Zhang and Wang, 2005). Arabidopsis leaves treated with simulated acid rain (SAR) show phenotypes similar to necrotic lesions caused by Pseudomonad infiltration, and genes known to be induced by the SA mediated pathogen resistance are up-regulated by acid rain (Lee et al., 2006). In this experiment, expression of 3 SA-mediated genes used in Lee et al. (2006); *enhanced disease susceptibility 1* (*EDS1*, At3g48090),

Table 2. Down-regulated genes by more than 3-fold upon 2 h acid (pH 3.0) treatment

Gene ID	Fold change 2h treatment	Fold change 12h treatment	Name
Abiotic stress-related			
AT4G26010	−6.6	1.0	Peroxidase
AT3G01190	−6.2	−2.2	Peroxidase
AT5G67400	−4.2	−1.3	Peroxidase
AT3G49960	−4.0	−2.0	Peroxidase
AT1G05240	−3.8	−1.4	Peroxidase
AT3G53250	−3.6	−1.1	Unknown
AT1G29430	−3.2	−1.4	Unknown
AT5G64100	−3.1	−1.3	Peroxidase
Gene expression-related			
AT5G25810	−3.9	1.0	TNY-DNA binding transcription factor
AT2G29660	−3.1	−1.1	Unknown (zinc ion binding)
Structural constituent of cell wall (extensin)			
AT3G54580	−8.0	−1.7	Cell wall organization and biogenesis
AT3G28550	−5.2	1.0	Cell wall organization and biogenesis
AT3G54590	−4.8	1.0	ATHRGP1 (Hydroxyproline- rich glycoprotein 1)
AT5G06630	−4.5	−1.2	Cell wall organization and biogenesis
AT5G35190	−4.4	−1.3	Cell wall organization and biogenesis
AT2G24980	−4.1	−1.5	Cell wall organization and biogenesis
AT5G06640	−4.0	1.0	Cell wall organization and biogenesis
AT3G62680	−3.8	−1.3	PRP3 (Proline-rich protein 3)
AT1G26250	−3.7	−1.3	Cell wall organization and biogenesis
AT1G23720	−3.4	1.0	Cell wall organization and biogenesis
AT4G08410	−3.2	1.1	Cell wall organization and biogenesis
Metabolism-related			
AT4G12510	−5.1	−1.1	Lipid binding
AT4G26530	−4.9	1.0	Fructose-bisphosphate aldolase
AT4G25820	−3.7	−1.3	XTR9 (Xyloglucan endotransglycosylase 9)
AT5G44440	−3.4	2.1	Electron carrier
AT1G64170	−3.2	1.1	ATCHX16 (Cation/H + exchangers)
AT4G37160	−3.2	−2.0	SKS15 (Copper ion binding)
AT5G01220	−3.1	1.5	SQD2 (Sulfoquinovosyldiacyl glycerol 2)
AT4G19690	−3.1	−2.4	IRT1 (Cadmium, iron ion transporter)
AT4G17340	−3.0	−1.2	DELTA-TIP2/TIP2 (water channel)
Unknown			
AT5G48430	−9.2	−1.7	Unknown
AT5G40590	−6.0	−1.5	Unknown
AT5G56540	−3.6	−1.1	AGP14 (Arabinogalactan protein 14)
AT2G44380	−3.3	−1.3	Unknown
AT4G26320	−3.3	1.0	AGP13 (Arabinogalactan protein 13)
AT3G02240	−3.2	−1.7	Unknown
AT2G33830	−3.2	1.3	Unknown
AT2G44370	−3.0	−1.1	Unknown

Table 3. Regulation of SA-mediated pathogen resistance genes by acid (pH 3.0) treatment

Gene ID	Fold change 2h treatment	Fold change 12h treatment	Name
AT2G14610	1.5	2.8	PR 1 (Pathogen-related 1)
AT3G48090	1.4	7.0	EDS 1 (enhanced disease susceptibility 1)
AT1G64280	1.6	1.7	NPR 1 (nonexpresssor of PR 1)
AT4G16890	−1.7	2.9	SNC 1 (Suppressor of NPR1)

nonexpresssor of PR (*NPR1*, At1g64280), *pathogen-related 1* (*PR1*, At2g14610), were also up-regulated 40%, 60%, 50%, respectively compared to control at 2 h acid treatment (Table 3). However, no significant increase in gene expression was observed after 12 h treatment (Table 3). Consist with above data, *suppressor of NPR*1 (*SNC1*, At4g16890) was down-regulated 1.7 times at 2 h, however, up-regulated 2.9 times at 12 h treatment (Table 3). It indicates *NRR1*, one of SA-mediated defense response gene can be up-regulated at 2 h acid treatment by *SNC*1 and can be reduced to the normal level by increased level of snc1 at 12 h. Therefore, it is supposed that SA-mediated signaling pathway is working against SAR and acid stress as well as biotic stresses.

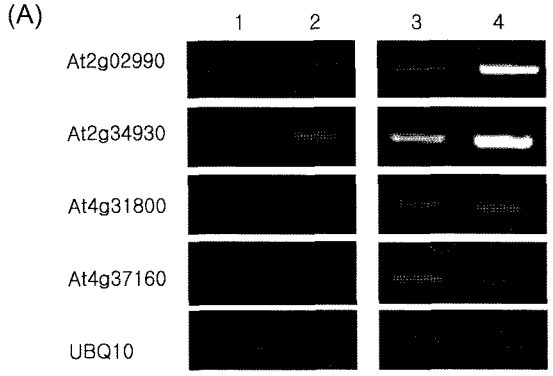
Among down-regulated, many family members of peroxidase were included, which down-regulate H_2O_2 level in plant cells. Usually peroxidases are highly expressed in response to drought and heat stress (Rizhskyc et al., 2004). In this experiment, we found some family members of peroxidase showed much down-regulation of mRNA. The similar results were reported earlier. When heat stress was applied, a few peroxidase genes showed up-regulation and some others showed reduced expression (Koussevitzky, 2008). Also DNA damaging agent, methyl methanesulfonate treatment also down-regulates expression of a peroxidase (At4g30170) more than 10-folds (Kim, 2006). However, the same gene, At4g30170 was not down-regulated by acid stress, indicating that family members of peroxidases may have different roles and expression patterns against different stresses. Among genes down-regulated in this study, genes encoding cell wall proteins, extensins were included. Extensins belong to multi-gene family that have been shown to be up-regulated by pathogen attack and wounding, and were presumed to reinforce plant cell wall (Showalter and Rumeau, 1990; Tiré et al., 1994). Some extensin genes can be induced by stresses such as wounding and pathogens (Merkouropoulos et al., 1999). other extensins such as At3g54580 and At3g28550 known to be down-regulated by hypoxia (Branco-Price et al., 2005), were also down-regulated by acid stress. However, collective down-

Table 4. Up-regulated genes by more than 3-fold upon 12 h acid (pH 3.0) treatment

Gene ID	Fold change		Name
	12h treatment	2h treatment	
Abiotic stress-related			
AT2G02990	7.4	3.5	RNS1 (Ribonuclease 1)
AT2G29500	5.6	2.8	Putative small heat shock protein
AT1G80820	3.7	7.0	CCR2 (Cinnamoyl CoA reductase)
AT2G32250	3.0	1.7	Unknown
Defense-related			
AT2G34930	5.8	4.8	Unknown (protein binding)
AT4G37990	3.4	4.2	ELI3-2 (Elicitor-activated gene 3)
AT1G61560	3.5	1.7	MLO6 (Mildew resistance locus O 6)
Metabolism-related			
AT1G26380	5.3	3.1	electron carrier
AT5G65280	5.0	1.6	Unknown (Catalytic activity)
AT1G68690	3.2	1.6	Protein kinase
Unknown			
AT5G54850	3.5	1.5	Unknown
AT1G12750	3.1	1.4	Unknown

Table 5. Down-regulated by more than 2-fold upon 12 h acid (pH 3.0) treatment

Gene ID	Fold change		Name
	12h treatment	2h treatment	
Abiotic stress-related			
AT1G75580	−2.3	−1.5	Calmodulin binding protein
AT3G01190	−2.2	−6.2	Peroxidase
AT3G49960	−2.0	−4.0	Peroxidase
Metabolism-related			
AT4G19690	−2.4	−3.1	IRT1 (Ion transporter)
AT3G62280	−2.2	−2.1	Carboxylic ester hydrolase
AT4G37160	−2.0	−3.2	SKS15 (SKU5 Similar 15)
Unknown			
AT1G07840	−3.3	1.5	Unknown
AT2G37380	−2.7	−2.6	Unknown
AT4G39200	−2.6	−1.5	Unknown (Structural constituent of ribosome)



(B)

Gene ID	Fold change			
	2h treatment		12h treatment	
	Microarray	RT-PCR	Microarray	RT-PCR
AT2G02990	3.5	4.0	7.4	6.5
AT2G034930	4.8	5.0	5.8	8.3
AT4G31800	3.4	4.1	1.2	1.1
AT4G37160	−3.2	−4.0	−2.0	−2.3

Fig. 3. RT-PCR analyses verifying microarray data. Plants were treated with acid (pH 3) for 2 and 12 h, respectively. (A) Expression levels of 4 genes were investigated by semi-quantitative RT-PCR. 1, 2 h treatment control (pH 5.6); 2, 2 h acid treatment (pH 3.0); 3, 12 h treatment control (pH 5.6); 4, 12 h acid treatment (pH 3.0). UBQ10 RT-PCR was used as the internal standard. (B) Comparison of microarray and semi-quantative RT-PCR data of 4 genes. For quantitative analyses of RT-PCR products, the intensities of EtBR-stained PCR products in Fig. 3A were scanned and quantified using the Scion Image programme (Scion Corporation, Frederick, MD, USA) and normalized to the UBQ10 band intensity at each condition.

regulation of so many members of peroxidase and extensin genes was very specific phenomenon to plants subjected to acid treatment, considering normal response to pathogen and wounding is up-regulation of these genes. Except a ribonuclease (*RNS1*, At2g02990), all abiotic stress-related genes which were up-regulated more than 3-fold by 2 h treatment, showed reduced levels of transcripts after 12 h treatment (Table 1). Therefore, this ribonuclease may play an important role thorough long-term acid stress with other genes whose levels were increased after 12 h than 2 h acid treatments (Table 4). Among genes down-regulated after 12 h acid treatment, two peroxidase genes (AT3G01190 and AT3G49960) which showed more than 3-fold down-regulation at 2 h, showed more than a 2-fold reduction in transcript level (Table 5). However, extensin members which showed more than 3-fold reduction at 2 h acid treatment, showed a relative increase in levels of transcripts at 12 h acid treatment (Table 2).

Overall, Arabidopsis showed very specific responses to acid treatment by down-regulating many members of peroxidase and extensin gene families. That response was different from plant response to wounding or pathogen

attacks. Acid treatment also turned on genes involved in signaling against other stresses, especially those related to wounding, which is not surprising when considering that acid rain directly causes damage to cuticle layers and necrosis on Arabidopsis leaves (Park and Lee, 1999). To validate these microarray results, we carried out semi-quantitative RT-PCR. Several induced and reduced genes were selected and subjected to RT-PCR (Fig. 3A). The band intensities of ethidium bromide-stained RT-CPR products in Fig. 3A were normalized to those of UBQ10 and the quantitative changes of gene expressions were compared to microarray data (Fig. 3B). The quantitative data of selected genes showed similar trend of gene expression and positive correlation with microarray data, confirming microarray data (Fig. 3B). Thus, we believe these microarray data will help to provide basic knowledge about plant response to acid stress and to develop acid stress-resistant plants.

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References

Bell, E. and Mullet, J. E. 1991. Lipoxygenase gene expression is modulated in plants by water deficit, wounding, and methyl jasmonate. *Mol. Gen. Genet.* 230:456-462.

Branco-Price, C., Kawaguchi, R., Ferreira, R. B. and Bailey-Serres, J. 2005. Genome-wide analysis of transcript abundance and translation in Arabidopsis seedlings subjected to oxygen deprivation. *Ann. Bot. (Lond)* 96:647-660.

Brosché, M. and Strid, A. 2003. Molecular events following perception of ultraviolet-B radiation by plants. *Physiol. Plant.* 117:1-10.

Choi, J. W., Kim, G. B., Huh, Y. C., Kwon, M. R., Mok, I. G., Kim, J. W., Lee, T. S., Kim, S. and Im, K. H. 2004. Cloning of genes differentially expressed during the initial stage of fruit development in melon (*Cucumis melo* cv. Reticulatus). *Mol. Cells* 17:237-241.

Delhaize, E. and Ryan, P. R. 1995. Aluminum toxicity and tolerance in plants. *Plant Physiol.* 107:315-321.

Derikvand, M., Sierra, J. B., Ruel, K., Pollet, B., Do. C., Thévenin. J., Buffard, D., Jouanin, L. and Lapierre, C. 2008. Redirection of the phenylpropanoid pathway to feruloyl malate in *Arabidopsis* mutants deficient for cinnamoyl-CoA reductase 1. *Planta* 227:943-956.

Evans, L. S. and Curry, T. M. 1979. Differential responses of plant foliage to simulated acid rain. *Am. J. Bot.* 66:953-962.

Foy, C. D. 1984. Physiological Effects of Hydrogen, Aluminum and Manganese Toxicities in Acid Soil. In: *Soil Acidity and Liming,* ed. by R. W. Pearson and F. Adams, pp. 57-97. Amer. Soc. Agronomy, Madison, Wisconsin, USA.

Foy, C. D. 1988. Plant adaptation to acid, aluminum-toxic soils. *Commun. Soil Sci. Plant Anal.* 19:959-987.

Kim, Y. S. 2006. Analysis of gene expression upon DNA damage in *Arabidopsis*. *J. Plant Biology* 49:298-302.

Koussevitzky, S., Suzuki, N., Huntington, S., Armijo, L., Sha, W., Cortes, D., Shulaev, V. and Mittler, R. 2008. Ascorbate peroxidase 1 plays a key role in the response of Arabidopsis thaliana to stress Combination. *J. Biol. Chem.* 283:34197-34203.

LeBrasseur, N. D., MacIntosh, G. C., Pérez-Amador, M. A., Saitoh, M. and Green, P.J. 2002. Local and systemic wound-induction of RNase and nuclease activities in Arabidopsis: RNS1 as a marker for a JA-independent systemic signaling pathway. *Plant J.* 29:393-403.

Lee, Y., Park, J., Im, K. H., Kim, K., Lee, J., Lee, K., Park, J. A., Lee, T. K., Park, D. S., Yang, J. S., Kim, D. and Lee, S. 2006. Arabidopsis leaf necrosis caused by simulated acid rain is related to the salicylic acid signaling pathway. *Plant Physiol. Biochem.* 44:38-42.

Melan, M. A., Dong, X., Endara, M. E., Davis, K. R., Ausubel, F. M. and Peterman, T. K. 1993. An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid, and methyl jasmonate. *Plant Physiol.* 101:441-450.

Merkouropoulos, G., Barnett, D. C. and Shirsat, A. H. 1999. The Arabidopsis extensin gene is developmentally regulated, is induced by wounding, methyl jasmonate, abscisic and salicylic acid, and codes for a protein with unusual motifs. *Planta* 208:212-219.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum* 15:473-497.

Ocampo, C. A., Moerschbacher, B. and Grambow, H. J. 1986. Increased lipoxygenase activity is involved in the hypersensitive response of wheat leaf cells infected with avirulent rust fungi or treated with fungal elicitor. *Z. Naturforsch* 41:559-563.

Park, J. B. and Lee, S. C. 1999. Growth responses of *Arabidopsis thaliana* exposed to simulated acid rain. *J. Kor. Soc. Hort. Sci.* 41:653-656.

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S. and Mittler, R. 2004. When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. *Plant Physiol.* 134:1683-1696.

Showalter, A. M. and Rumeau, D. 1990. Molecular biology of the plant cell wall hydroxyproline-rich glycoproteins. In: *Organization and assembly of plant and animal extracellular matrix,* ed. by W. S. Adair and R. P. Mecham, pp. 247-281. Academic Press, New York. USA.

Tiré, C., Rycke, R. D., Loose, M. D., Inzé, D., Montagu, M. V. and Engler, G. 1994. Extensin gene expression is induced by mechanical stimuli leading to local cell wall strengthening in *Nicotiana plumbaginifolia*. *Planta* 195:175-181.

Zhang, Y. and Wang, L. 2005. The WRKY transcription factor superfamily: its origin in eukaryotes expansion in plants. *BMC Evol. Biol.* 5:1-12.