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e-Xtra*

Identification of 118 *Arabidopsis* Transcription Factor and 30 Ubiquitin-Ligase Genes Responding to Chitin, a Plant-Defense Elicitor

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Chitin, found in the cell walls of true fungi and the exoskeleton of insects and nematodes, is a well-established elicitor of plant defense responses. In this study, we analyzed the expression patterns of *Arabidopsis thaliana* transcription factor (TF) and ubiquitin-ligase genes in response to purified chitooctaose at different treatment times (15, 30, 60, 90, and 120 min after treatment), using both quantitative polymerase chain reaction and the Affymetrix *Arabidopsis* whole-genome array. A total of 118 TF genes and 30 ubiquitin-ligase genes were responsive to the chitin treatment. Among these genes, members from the following four TF families were overrepresented: APETALA2/ethylene-reponsive element binding proteins (27), C2H2 zinc finger proteins (14), MYB domain-containing proteins (11), and WRKY domain transcription factors (14). Transcript variants from a few of these genes were found to respond differentially to chitin, suggesting transcript-specific regulation of these TF genes.

Additional keyword: PAMP

In nature, plants are confronted by a variety of pathogens and pests. As part of their innate immune system, plants recognize these invaders by virtue of pathogen-associated molecular patterns (PAMP), cellular substituents common to a variety of pathogens (Nürnberger et al. 2004). PAMP include such molecules as flagellin (Felix et al. 1999; Gomez-Gomez 2004) and various oligosaccharides, including chitin. Recognition of the PAMP results in the induction (elicitation) of various pathogen defense pathways in the plant.

Recognition of pathogen elicitors by the plant can induce hypersensitive cell death and synthesis of antimicrobial molecules (phytoalexins; Heath 2000). For example, volicitin (Schmelz et al. 2003) and flagellin (Zipfel et al. 2004) activate the pathways mediated by jasmonic acid (JA) and ethylene (ET), two plant hormones with a major role in plant pathogen defense. Activation of these pathways also results in the synthesis of a variety of proteins (pathogenesis-related [PR] proteins) thought to play a role in pathogen defense. One such example is chitinase, an enzyme that catalyzes the hydrolysis of chitin polymers, composed of β-1→4-linked *N*-acetylglucosamine, found in the cell walls of pathogenic fungi and in the exoskeleton of insects and nematodes (Shibuya and Minami 2001). Application of purified chitin oligomers from different sources (such as yeast and crab shell) to plants or their cell cultures was shown to elicit various defense-related reactions, such as activation of PR genes and synthesis of phytoalexins (Shibuya and Minami 2001; Stacey and Shibuya 1997). DNA microarray analysis showed a large number of *Arabidopsis thaliana* genes induced upon chitin elicitation (Ramonell et al. 2002, 2005; Zhang et al. 2002). Comparison of the response of wild-type *Arabidopsis* with various mutants blocked in the ET, salicylic acid (SA), and JA signaling pathways showed that the initial response to chitin elicitation was independent of these pathways, suggesting a unique pathway for chitin action (Zhang et al. 2002). Initial activation of a mitogen-activated protein kinase cascade is important to chitin elicitation (Wan et al. 2004). A criticism often leveled against research dealing with PAMP is that elicitor action is not always related to pathogen virulence. However, in the case of chitin, Ramonell and associates (2005) showed that mutations in some genes identified by microarray analysis as responsive to chitin resulted in increased susceptibility to the powdery mildew fungal pathogen *Erysiphe cichoracearum*. Chitin pretreatment of plants also reduces susceptibility to subsequent fungal pathogen challenge (Tanabe et al. 2006). Thus, chitin elicitation appears to play a significant role in plant defense to fungal pathogens.

Structure-function analysis in a variety of plants showed that larger chitin oligomers (degree of polymerization (*d.p.*) = 7 to 8 *N*-acetylglucosamine residues) were most effective as elicitors (Shibuya and Minami 2001; Stacey and Shibuya 1997). A number of membrane-associated chitin-binding proteins, presumably chitin receptors, were previously identified in a variety of plants, such as soybean (Day et al. 2001), French bean (Bindschedler et al. 2006), and tomato (Baureithel et al. 1994). Recently, Kaku and associates (2006) identified the likely chitin receptor in rice as a protein with one transmembrane domain and with two predicted, extracellular LysM domains. Extracellular LysM domains are also found in those proteins predicted to be the plant receptors for the lipo-chitin nodulation factors produced by rhizobium and essential for legume nodulation (Stacey et al. 2006).

The fact that chitin elicits de novo gene expression suggests the involvement of transcription factors (TF). DNA microarray studies have greatly expanded the list of TF that are responsive to pathogen inoculation and other treatments. Among the tran-

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Microarray sequence data has been deposited in the NCBI Gene Expression Omnibus database. The accession number for the data set is GSE4746.

*The *e*-Xtra logo stands for "electronic extra" and indicates supplemental material is published online. Seven additional tables are available online.

scription factor families implicated in plant disease resistance are the AP2-ERE (Kim et al. 2000; McGrath et al. 2005), C2H2 zinc finger (McGrath et al. 2005), MYB (Hui et al. 2003; Liu et al. 2004; McGrath et al. 2005; Yang and Klessig 1996; Vailleau et al. 2002), WRKY (Hui et al. 2003; Kim and Zhan 2004; Liu et al. 2004, 2005; McGrath et al. 2005; Park et al. 2006; Ramonell et al. 2005; Ryu et al. 2006; Wan et al. 2004), GRAS (Day et al. 2003, 2004), bZIP (Thurow et al. 2005), NAC domain-containing (McGrath et al. 2005), Whirly (Desveaux et al. 2005), DOF (McGrath et al. 2005; Yanagisawa 2002), and MYC (Boter et al. 2004; Lorenzo et al. 2004) families. However, mRNAs for TF are often present in relatively low levels in cells, and DNA microarray hybridizations do a poor job in accurately measuring such low abundant mRNAs. To address these issues, Czechowski and associates (2004) designed 2,297 quantitative polymerase chain reaction (qPCR) primers for all the potential 2,077 TF genes and 150 putatitve ubiquitin-ligase genes in *A. thaliana.* Their results suggest that qPCR measurement of TF expression is at least 100-fold more sensitive than DNA microarray hybridization (Czechowski et al. 2004; McGrath et al. 2005).

In our current study, we utilized the *Arabidopsis* TF qPCR primer set for transcriptional profiling of TF gene expression after elicitation with chitooctaose (*d.p.* = 8). These results were directly compared with data derived from hybridization to the Affymetrix *Arabidopsis* genome array. In addition to TF genes, we also examined the response of some ubiquitin-ligase genes to chitin, due to their probable role in plant defense (Liu et al. 2002; Yang et al. 2006). Collectively, these methods identified 118 TF genes and 30 ubiquitin-ligase genes to be responsive to chitooctaose. During the analysis, it became apparent that some genes expressed more than one transcript and, in a few such cases, a transcript-specific response to chitooctaose was seen.

RESULTS

Arabidopsis genes regulated by chitooctaose: a summary.

We sought to identify the TF genes involved in the response to chitooctaose, a main elicitor of the plant defense response against pathogens. In order to establish the most complete list of chitin-responsive TF genes, we used two different technologies, high-throughput (quantitative reverse transcription) qRT-PCR and microarray hybridization. Collectively, these two technologies allowed us to identify 118 *Arabidopsis* TF genes responding after 15 or 30 min of chitooctaose treatment. These 118 TF genes represent 29 TF families. The same experiments identified 30 ubiquitin-ligase genes as responsive to chitin elicitation. As described below, these gene lists were compiled not only by comparison of both the qRT-PCR and microarray results but also by resolving discrepancies between the two data sets.

TF responding to chitooctaose.

We measured the expression profile of 2,077 *Arabidopsis thaliana* TF genes by qRT-PCR, utilizing the 2,146 primer set library (Czechowski et al. 2004). Three independent experiments were done using *Arabidopsis* seedlings treated with chitooctaose or mock-treated with water for 15 or 30 min. These experiments identified 99 TF that responded significantly to chitooctaose elicitation (Table 1) (the complete set of qRT-PCR data from these experiments is available as Supplemental Table I).

Using the same RNA as used above, we also profiled the transcriptional response of *Arabidopsis* to 30 min of chitooctaose treatment utilizing the *Arabidopsis* Affymetrix DNA microarray. This data set identified an additional 19 *Arabidopsis* TF genes as responsive to chitooctaose treatment. Microarray results were confirmed by qRT-PCR analysis using RNA samples from 15 and 30 min after chitin treatment (Table 1).

Taken together, out of the 118 genes identified by the high-throughput qRT-PCR and Affymetrix hybridization, 61 and 106 TF genes were significantly down- or up-regulated (*P* value <0.05 and fold change <0.5 or >2) 15 and 30 min after chitooctaose treatment (Table 1). These chitooctaose-responsive genes represent 19 and 26 different families, respectively, based on their functional domains. Most of the responsive genes were up-regulated after chitooctaose treatment (59 and 101, respectively, after 15 and 30 min of chitin treatment). A total of 49 TF genes were up-regulated both 15 and 30 min after chitooctaose treatment.

When compared with the gene family distribution of the primer set library used for qRT-PCR expression analysis (we consider this library as representative of the TF genes existing in *Arabidopsis*; 75 different families with nine families representing more than 50% of the library [Fig. 1, top; Supplemental Table II]), four TF families were significantly overrepresented in the 118 TF genes that responded to chitin treatment. These included the APETALA2/ET-reponsive element binding protein (AP2/ERE), C2H2 zinc finger, MYB domain transcription factor, and WRKY domain transcription factor families. These four families represent 28.8% of the qPCR primer library (Fig. 1, top) but represent 62.3% (38 TF genes) of those that responded after 15 min of treatment and 57.6% (61 TF genes) of those responding after 30 min (Fig. 1, bottom).

Chitooctaose strongly affects TF gene expression after 30 min of treatment.

The results obtained above were generated from plants treated 15 and 30 min after chitooctaose treatment. In order to extend these observations and to confirm the results, independent qRT-PCR experiments were performed, using *Arabidopsis* plants treated with chitooctaose for 60, 90, or 120 min. Analyses were performed on 41 TF genes, selected from the list of 118 genes according to their fold change and their family membership (Supplemental Table III). According to the National Center for Biotechnology Information (NCBI) database, two to three transcript variants exist for four of these 41 genes (*AT4G31550* (*WRKY11*), *AT2G23320* (*WRKY15*), *AT5G59780* (*MYB59*), and *AT3G54810* (*C2C2(Zn)* GATA transcription factor). Therefore, when possible, primers were designed that would distinguish between the various transcripts. All the primers were compared against the NCBI database to check their specificity.

Respectively, 39 and two TF genes were found significantly up- and down-regulated, respectively, by chitooctaose treatment at one or more of the five timepoints tested (Fig. 2; Supplemental Table IV). The results clearly show the maximum response to chitooctaose occurs 30 and 60 min after chitin treatment and less often after 15 min of chitin treatment (Fig. 2). These data are consistent with previous results (Ramonell et al. 2005).

Expression of the AP2/ERE, MYB, WRKY, and GRAS gene families.

As mentioned above, four gene families were overrepresented among the chitooctaose-responsive TF genes (i.e., AP2/ERE, C2H2 zinc finger, MYB, and WRKY families). We focused on selected members of AP2/ERE, MYB, and WRKY families to better characterize their response during the first 2 h of chitooctaose elicitation. In addition, because rice GRAS family members were implicated in the response to chitin (Day et al. 2003), we also selected a few *Arabidopsis* GRAS genes for this analysis. Specifically, we selected seven (*AT1G22810*, *AT1G64380*, *AT3G23230*, *AT5G51190*, *AT4G17490*, *AT5G47230*, *AT5G61590*) from the AP2/ERE family, two

(*AT1G18570*, *AT5G59780*) from MYB, six (*AT2G23320*, *AT2G46400*, *AT4G23810*, *AT4G31550*, *AT5G15130*, *AT2G38470*) from WRKY, and four (*AT1G07520*, *AT3G46600*, *AT4G17230*, *AT5G59450*) from the GRAS family (Fig. 2).

The members of the AP2/ERE and MYB TF family were up-regulated 2.2- (*AT5G47230*, 60 min) to 171-fold (*AT3G23230*, 60 min) and 19-fold (*AT1G18570*, 60 min), respectively. Some genes were down-regulated. For example, members of the AP2/ERE and MYB families were repressed 2.2- (*AT5G61590*, 30 min) and 4.8-fold (*AT5G59780*, 30 min).

The selected members of the GRAS and WRKY TF families were exclusively up-regulated after chitin treatment (the maximum response for each gene was, respectively, between 3.6- [*AT1G07520*, 60 min] to 4.7-fold [*AT4G17230*, 60 min] and

Table 1. The expression of 118 *Arabidopsis* transcription factor (TF) genes respond to chitooctaose treatment[a]

		Response after 15 min		Response after 30 min	
Gene family	**Gene ID**	***t* Test**	**Fold Change**	***t* Test**	**Fold Change**
TF genes identified by high-throughput qPCR reaction					
AP2/ERE	AT1G21910	0.00091	2.1		
	AT1G22810	0.00430	39.2	0.00293	91.9
	AT1G28370	0.00035	3.9	0.00822	6.7
	AT1G33760	0.00436	3.9	0.00063	7.4
	AT1G53170			0.04880	2.1
	AT1G64380	0.00279	3.5		
	AT1G68840	0.00994	3.1	0.00728	3.0
	AT1G71520			0.03147	101.6
	AT1G75490	0.00710	2.3		
	AT1G77640			0.04020	2.4
	AT2G31230			0.00084	3.8
	AT2G33710	0.03310	5.9	0.00005	18.2
	AT2G44840	0.00671	17.1	0.00038	37.5
	AT3G15210	0.00401	2.6	0.02988	2.7
	AT3G23250			0.01389	9.1
	AT3G50260			0.00020	3.4
	AT4G17500	0.01168	3.6	0.00200	4.4
	AT4G18450	0.00942	3.6	0.00006	4.3
	AT4G28140			0.01630	3.7
	AT4G34410	0.00000	4.3	0.00165	5.7
	AT5G47220	0.02225	2.8	0.00265	3.9
	AT5G61590			0.00711	***0.5***
	AT5G61600	0.00557	9.5	0.00554	8.3
ARR-A class transcription factor	AT1G19050			0.01684	2.3
Aux/IAA	AT1G04100			0.03550	2.5
bHLH,Basic Helix-Loop-Helix	AT1G32640			0.00792	2.6
	AT3G56770	0.01772	2.1		
	AT5G56960			0.01048	15.6
bZIP transcription factor	AT2G34600	0.02466	7.9		
	AT2G36270			0.04707	2.1
	AT4G29110			0.01886	3.2
	AT2G17770			0.00736	16.8
C2C2(Zn) CO-like, Constans-like zinc finger	AT3G21150	0.01040	3.2	0.00320	14.6
	AT4G39070			0.00769	2.2
C2C2(Zn) DOF zinc finger	AT1G51700	0.00028	4.6	0.00229	14.3
C2H2 zinc finger	AT1G08930			0.00159	3.7
	AT1G27730	0.00012	10.5	0.00024	17.2
	AT1G68360			0.02558	2.7
	AT2G37430	0.00001	34.9	0.00017	78.6
	AT3G19580			0.00196	10.3
	AT3G46080			0.00697	7.4
	AT3G46090	0.00011	7.4	0.00189	11.0
	AT3G53600			0.00790	5.3
	AT5G04340			0.00036	11.9
	AT5G22890			0.01644	***0.5***
	AT5G43170	0.01205	2.2		
	AT5G59820			0.00120	13.3
	AT5G67450	0.00113	6.3	0.00119	17.1
C3H zinc finger	AT3G55980	0.00007	9.7	0.00003	14.0
CHP-rich	AT4G01350			0.01438	2.6
expressed protein	AT5G13220			0.01943	3.1
F-box protein	AT1G09650	0.01684	***0.4***		
GRAS transcription factor	AT1G07520			0.01073	3.4
	AT3G46600	0.00310	2.2	0.02495	3.3
	AT5G59450			0.04996	3.5
HB,Homeobox transcription factor	AT3G49530	0.00010	2.3	0.00529	3.7
HSF,Heat-shock transcription factor	AT1G67970	0.04882	2.1	0.02356	2.6
	AT2G26150			0.01421	2.2
	AT4G18880	0.01236	3.3	0.00154	9.0

Continued on next page

[a] *P* values associated with these experiments. Down-regulated genes are highlighted by showing values in bold italics, others are all up-regulated. The genes are grouped by gene family. Fold change shown is the average.

8.1- [*AT2G23320*, 60 min] to 31.3-fold [*AT4G23810*, 60 min]). Together with the results obtained from the primer library, we identified 14 WRKY genes that were significantly up-regulated 15 to 120 min after chitin treatment. To complete the analysis of the WRKY TF family, we compared the expression levels of the three transcript variants encoded by *AT4G31550* (*WRKY11*). At 15 min after chitooctaose treatment, *AT4G31550.3* was up-regulated more than transcript variant *AT4G31550.1* and *AT4G31550.2* (Fig. 2). At later timepoints, this difference was less noticeable (Fig. 2).

Expression of 30 putative ubiquitin-ligase genes is affected by chitooctaose treatment.

The ubiquitin-ligase genes are also known to play a role in the plant defense response (Liu et al. 2002; Yang et al. 2006). Therefore, predicted members of this family were also analyzed by

Table 1. *Coninued from preceding page*

		Response after 15 min		Response after 30 min	
Gene family	**Gene ID**	***t* Test**	**Fold Change**	***t* Test**	**Fold Change**
TF genes identified by high-throughput qPCR reaction (*continued*)					
HSF,Heat-shock transcription factor (*continued*)					
	AT5G03720			0.00029	3.5
	AT5G62020	0.00010	2.8	0.00008	5.4
JUMONJI	AT1G30810			0.02862	2.1
	AT5G46910			0.01840	2.4
MYB domain transcription factor	AT1G18570	0.00112	3.2	0.00185	5.6
	AT1G74650			0.00034	2.7
	AT3G50060			0.00116	3.3
	AT3G53200			0.01232	***0.4***
	AT4G28110	0.02977	11.8		
	AT4G37260	0.02652	14.1	0.00308	2.8
	AT5G23000			0.00046	3.0
	AT5G62470			0.00038	8.9
	AT5G67300			0.00362	2.0
	AT5G04760			0.00962	2.8
NAC domain transcription factor	AT1G34180			0.00079	2.4
	AT3G44350			0.00033	7.7
	AT2G17040	0.00135	3.7	0.00105	11.1
	AT2G18060	0.03570	2.0		
	AT5G08790			0.00166	2.5
	AT5G24590	0.02142	2.1	0.01225	3.3
NPR1-like	AT4G26120			0.02216	3.0
	AT5G45110	0.00907	2.8	0.02707	2.6
Others, DEAD/DEAH box helicase	AT1G19180			0.00694	4.2
PHD finger transcription factor	AT5G58610	0.01869	2.7		
Psudo ARR transcription factor	AT5G02810	0.02105	***0.5***		
SET-domain transcriptional regulator	AT2G35160			0.04507	2.2
Trihelix, Triple-Helix transcription factor	AT5G03680			0.02503	***0.4***
WHIRLY	AT2G13790			0.00370	3.3
	AT4G33050	0.00499	2.0	0.00187	4.8
	AT5G35735			0.00062	4.6
WRKY domain transcription factor	AT1G62300	0.01033	2.2	0.00135	4.1
	AT1G80840	0.00031	5.7	0.00462	21.9
	AT2G24570			0.02464	4.4
	AT2G38470	0.02319	5.5		
	AT3G56400	0.01673	2.8	0.02684	3.7
	AT4G01250	0.02327	2.3	0.01359	10.8
	AT4G23550			0.00494	2.7
	AT4G23810	0.00442	13.0	0.00897	28.8
	AT4G31800	0.00303	2.9	0.00014	5.7
	AT5G49520	0.02143	2.1	0.02704	5.6
TF genes identified by Affymetrix hybridization and confirmed by qRT-PCR					
AP2/ERE	AT3G23230			0.0006	57.1
	AT5G51190	0.0005	21.3	0.0002	17.8
	AT5G47230			0.0374	2.5
	AT4G17490	0.0001	14.0	0.0006	17.9
Argonaute	AT1G31290	0.0450	6.0	0.0068	7.1
bZIP transcription factor	AT1G42990	0.0004	2.5	0.0026	5.5
C2C2(Zn) GATA transcription factor	AT3G54810			0.0164	3.1
C2H2 zinc finger	AT3G28210			0.0030	2.2
	AT3G52800	0.0001	2.3	0.0004	3.3
C3H zinc finger	AT2G40140	0.0024	6.4	0.0007	9.0
expressed protein	AT1G17380			0.0076	6.7
GRAS transcription factor	AT4G17230	0.0020	2.4	0.0012	4.5
HSF,Heat-shock transcription factor	AT4G11660	0.0044	2.7	0.0149	2.5
MYB domain transcription factor	AT5G59780			0.0089	***0.2***
Transcriptional Adaptor Zinc Bundle (TAZ) domain	AT4G37610			0.0045	2.5
WRKY domain transcription factor	AT4G31550	0.0001	4.3	0.0004	10.0
	AT2G23320	0.0010	2.0	0.0066	3.7
	AT2G46400	0.0000	6.3	0.0001	10.9
	AT5G15130	0.0006	4.3	0.0002	6.4

qRT-PCR and by Affymetrix array hybridization starting with the same RNA used for TF gene transcriptional profiling. A total of 30 ubiquitin-ligase genes were found up- or down-regulated at either 15 or 30 min or both after chitooctaose treatment using both methods.

The high-throughput qPCR primer set library allowed us to measure the expression of 150 ubiquitin-ligase genes (Supplemental Table V). These experiments identified five and seven ubiquitin-ligase genes that were significantly up-regulated (P value <0.05 and fold change <0.5 or >2) 15 and 30 min, respectively, after chitooctaose treatment (Table 2). None of the ubiquitin-ligase genes were found down-regulated. Four ubiquitin-ligase genes were up-regulated both 15 and 30 min after chitooctaose treatment. Collectively, qRT-PCR analysis identified a total of eight ubiquitin-ligase genes that were significantly up-regulated 15 and 30 min after chitooctaose treatment

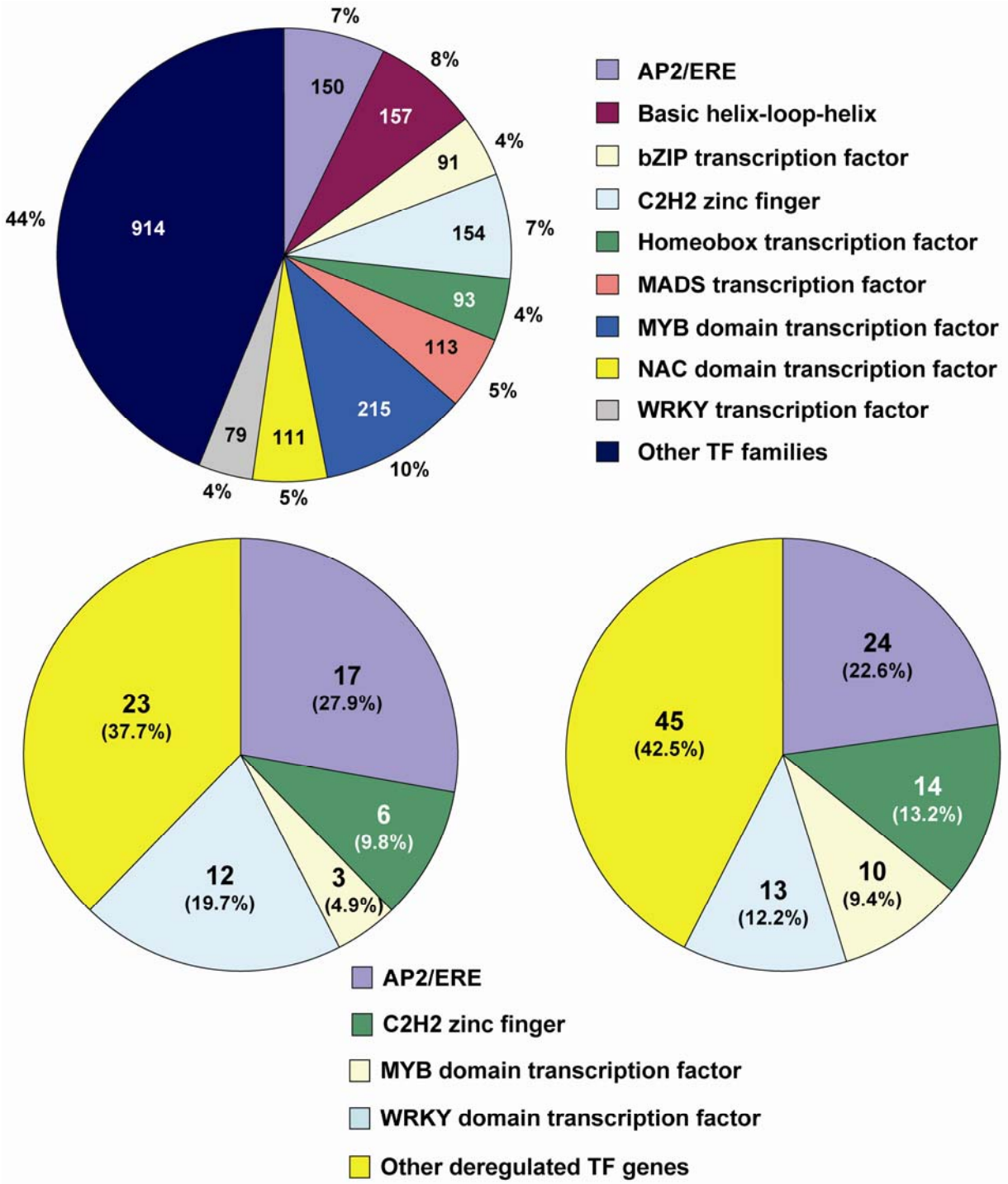

Fig. 1. Transcription factor (TF) genes whose expression responded to chitooctaose treatment as determined by high-throughput quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Top, Gene family distribution in the primer set library used for high-throughput TF gene expression analysis by qRT-PCR (we consider this library as including all the *Arabidopsis* TF genes). The 2,146 primer set allowed the expression analysis by RT-PCR of 2,077 *Arabidopsis* genes. These genes can be divided into 75 different families. This chart shows the distribution of the most abundant families represented in the number of genes in each family. Bottom left, gene families most responsive after 15 min of treatment and bottom right, those most responsive after 30 min of treatment. The number of genes responding significantly for each family is indicated. The percentage is calculated according to the total number of genes found to be responding significantly (118) and is not based on the total genes analyzed only by qRT-PCR or by Affymetrix DNA microarray hybridization.

(*AT1G74410*, *AT4G30370*, *AT5G38895*, *AT5G66070*, *AT1G23030*, *AT1G66160*, *AT3G52450*, and *AT5G64660*) (Table 2).

Analysis of the Affymetrix array data identified 26 ubiquitin-ligase genes that were chitooctaose responsive (P value <0.05, fold change >2 or <0.5; two were down-regulated and 24 were up-regulated after chitin treatment). However, the expression of only 16 of these 26 genes could be confirmed using the initial qRT-PCR primer set (Czechowski et al. 2004). The qRT-PCR primers for the remaining 10 genes were redesigned and were

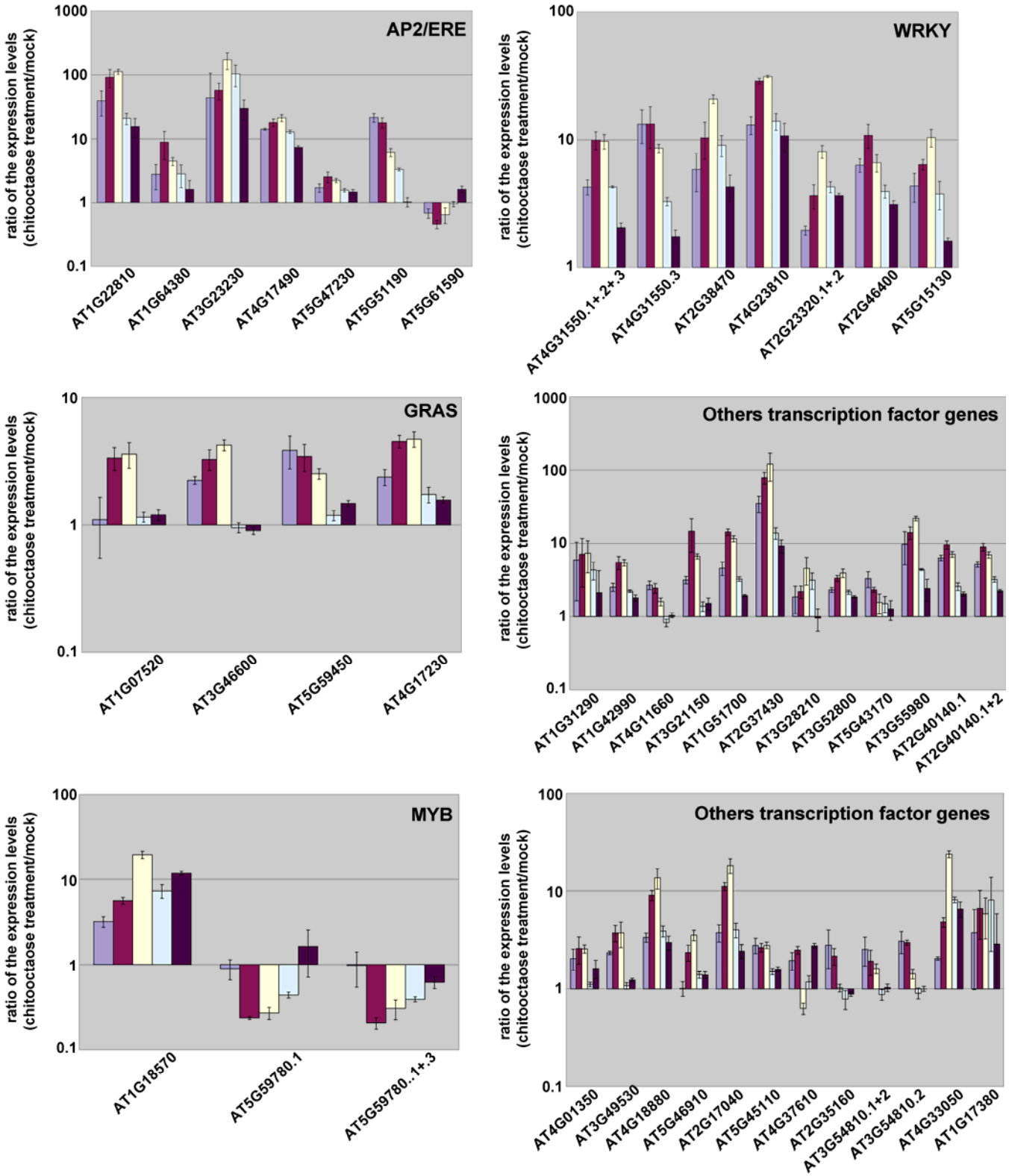

Fig. 2. Analysis of the expression levels of 41 *Arabidopsis thaliana* transcription factor (TF) genes after chitooctaose treatment. A subset of the TF genes found to respond to chitooctaose by high-throughput quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis and microarray hybridization were selected for this study. Using a 96-well format, qRT-PCR reactions were performed on cDNA isolated from plants treated with chitooctaose (left to right) 15 (blue), 30 (purple), 60 (yellow), 90 (blue light), and 120 (dark purple) min. The gene designations are shown on the x axis while the y axis presents the ratio of the expression levels (chitooctaose treatment/mock).

used to test for expression in response to chitin elicitation. Overall, we were only able to confirm the response of 22 of the 26 genes identified by microarray analysis using qRT-PCR (one down-regulated and 21 up-regulated) (Table 2). Collectively, the qRT-PCR data and Affymetrix data identified 30 ubiquitin-ligase genes that responded to chitooctaose elicitation.

To analyze the expression kinetics of selected ubiquitin-ligase genes, qRT-PCR experiments were performed on 11 ubiquitin-ligase genes using *Arabidopsis* plants treated with chitooctaose during 15, 30, 60, 90, and 120 min. Two of the 11 genes analyzed by qRT-PCR (*AT2G04240* and *AT5G48655*) encode different transcript variants. When possible, primers were designed to amplify specifically one transcript variant. Respectively, 10 and 1 ubiquitin-ligase genes were found significantly up- and down-regulated by chitooctaose treatment at one or more of the five timepoints tested (Fig. 3). Similarly to the response of the TF genes to chitin treatment, ubiquitin-ligase genes are the most regulated by chitooctaose after 30 and 60 min of treatment (Fig. 3).

qPCR analysis revealed that transcript variant *AT5G48655.3* was strongly up-regulated at the different timepoints (Fig. 3). Concerning *AT2G04240*, the transcript variant *AT2G04240.2* was down-regulated 15 and 30 min after chitin treatment, in comparison to *AT2G04240.1* (Fig. 3). Interestingly, for each of these genes, the expression level of one transcript variant was more affected by chitin than the others, especially 30 min after treatment (*AT5G48655.3*, *AT2G04240.2*) (Fig. 3).

Table 2. The expression of 30 ubiquitin-ligase genes respond to chitooctaose treatment[a]

	15 min		30 min	
Gene ID	***t* Test**	**Fold Change**	***t* Test**	**Fold Change**
Genes identified by high-throughput qPCR reaction				
AT1G23030	0.0141	2.0		
AT1G66160	0.0014	7.4	0.0037	4.5
AT1G74410			0.0487	2.4
AT3G52450	0.0075	7.3	0.0186	9.6
AT4G30370	0.0307	3.0	0.0279	3.9
AT5G38895			0.0307	2.8
AT5G64660			0.0005	6.2
AT5G66070	0.0076	3.6	0.0008	5.1
Genes identified Affymetrix hybridization and confirmed by qRT-PCR				
AT1G20823	…	…	0.0119	10.8
AT2G04240.1+2	…	…	***0.0115***	***0.6***
AT2G04240.2	…	…	***0.0130***	***0.3***
AT2G28830	…	…	0.0014	2.6
AT2G35930	…	…	0.0141	10.2
AT3G05200	…	…	0.0323	4.6
AT3G11840	…	…	0.0007	19.0
AT3G16720	…	…	0.0116	8.9
AT3G18710	…	…	0.0001	11.0
AT3G19380	…	…	0.0173	2.1
AT3G46620	…	…	0.0051	7.9
AT4G11360	…	…	0.0119	3.3
AT4G15975	…	…	0.0065	25.4
AT4G26400	…	…	0.0175	3.9
AT4G33940	…	…	0.0023	2.4
AT4G35480	…	…	0.0234	8.0
AT5G01830	…	…	0.0142	4.7
AT5G09800	…	…	0.0053	5.8
AT5G10380	…	…	0.0409	2.1
AT5G27420	…	…	0.0028	26.7
AT5G37490	…	…	0.0011	18.0
AT5G48655.1+.2+.3	…	…	0.2506	2.8
AT5G48655.3	…	…	0.0000	19.6
AT5G59550	…	…	0.0008	9.0

[a] The average ratio of chitooctaose/mock treatment between three independent replicates is shown for each of the ubiquitin-ligase genes identified by high-throughput quantititative polymerase chain reaction (PCR) reaction (15 and 30 min) and by Affymetrix hybridization and confirmed by quantitative reverse transcription-PCR (30 min). Also shown are the *P* values associated with these experiments. Down-regulated genes are highlighted by showing values in bold italics, others are all up-regulated.

Comparison of qRT-PCR reactions and Affymetrix Array results.

As discussed above, using the same RNA samples (30 min after chitooctaose treatment), high-throughput qRT-PCR and Affymetrix analysis of transcript abundance gave significantly different results for selected genes.

The Affymetrix *Arabidopsis* whole-genome array (ATH1) represents approximately 24,000 genes with 1,912 putative TF and ubiquitin-ligase genes represented. Using the DNA microarray analysis software DNA Chip Analyzer (dChip) (Li and Wong 2001a and b), 90 of the 1,912 TF and ubiquitin-ligase genes (4.7%) were found to be significantly responsive 30 min after chitin treatment (twofold, *P* value <0.05). By comparison, among the 94 (4.2% of the 2,227) genes found responsive to chitin by high-throughput qPCR at this timepoint, 85 genes are found on the Affymetrix array. A comparison of the microarray and qRT-PCR results showed that 52 of the 85 genes (61.2%) analyzed both by qRT-PCR and DNA microarray were confirmed by both methods. Totals of 33 and 38 genes, respectively, were identified either only by qRT-PCR or only by the Affymetrix arrays.

Because more than 50% of the TF and ubiquitin-ligase genes were found by both methods to be regulated by chitooctaose, qPCR and microarray hybridization are valuable complementary technologies to detect gene expression. However, what about those genes identified by only one of these methods? The average microarray spot intensity for those genes identified only by qRT-PCR was 341. In contrast, the average spot intensity for those genes found regulated by chitooctaose after microarray hybridization was 787. Therefore, the lack of detection of specific genes by the microarray is likely due to an inability to accurately measure the expression of low-abundance mRNA, as compared with qPCR. These results confirm the usefulness of qRT-PCR for exploring the expression of low-expressed genes.

On the other hand, as discussed before, subsequent qRT-PCR experiments were able to confirm the regulation by chitooctaose of 19 TF and 10 ubiquitin-ligase genes not identified by our initial high-throughput qPCR reactions. However, this confirmation required redesigning the primers used for qRT-PCR. Thus, use of the correct primer sequences was the crucial parameter to analyze the expression of these 29 genes.

Plant response to chitin depends on *d.p.*

Previous studies with a variety of plants showed that the response to chitin was dependent on the *d.p.*, with the larger chitin oligomers (*d.p.* = 7 to 8) being most effective (Day et al. 2001; Wan et al. 2004; Zhang et al. 2002). Therefore, we compared the response of the 41 TF genes and the 11 ubiquitin-ligase genes to either chitotetraose (*d.p.* = 4) or chitooctaose after 30 min of treatment. Chitotetraose treatment did not affect the expression of 37 TF and 10 ubiquitin-ligase genes (*P* value>0.05 or fold change >0.5 or <2) (Fig. 4, Supplemental Table VI). However, the expression of four TF genes (*AT4G23810*, *AT4G31550.3*, *AT2G37430*, *AT4G17490*) and one ubiquitin-ligase gene (*AT5G48655.3*) responded both to chitotetraose (*P* value <0.05 and fold change <0.5 or >2) (Fig. 4) and chitooctaose. Interestingly, expression of two transcript variants (*AT5G48655.3*, *AT4G31550.3*) was more affected than the others (Fig. 4). Nevertheless the expression of these five genes was less affected by chitotetraose than by chitooctaose treatment. Comparison of the expression level of these

genes between plants treated 30 min with chitooctaose and chitotetraose suggests that only those genes strongly expressed in response to chitooctaose also responded to chitotetraose. Therefore, it is likely that these results do not reflect a difference in specificity but merely a reflection of the sensitivity of some genes to chitin elicitation.

DISCUSSION

Chitin reprograms plant gene expression.

Chitin-responsive TF genes likely represent key elements in the ability of chitin to modify gene expression as part of the plant defense reaction. Indeed, previous microarray results showed that a large number of genes were affected more than 1.5-fold by chitooctaose treatment, with 1,070 genes up-regulated and 550 genes down-regulated (Ramonell et al. 2005). TF gene families previously implicated in the plant defense response are specifically affected by chitin. For example, the expression of 27 AP2/ERE, 15 C2H2 zinc finger, 11 MYB, 14 WRKY, and four *GRAS* TF genes were affected by chitooctaose. Members from these TF families were previously shown or implicated to modulate plant defense (Eulgem 2005; Eulgem et al. 2000; Gutterson and Reuber 2004). For example, *OsEREBP1* (Kim et al. 2000), an *Oryza sativa* member of the AP2/ERE gene family, and 16 *Arabidopsis* AP2/ERE genes were reported to respond to fungal inoculation (*Alternaria brassicicola*; McGrath et al. 2005). Likewise, the expression of five *AthC2H2* zinc finger genes (McGrath et al. 2005) responded to *Alternaria brassicicola*. In *Arabidopsis*, the expression of *AtMYB30* (Vailleau et al. 2002) and 12 other *Arabidopsis* MYB genes (McGrath et al. 2005) was affected by bacterial pathogens and *Alternaria brassicicola,* respectively. In tobacco, one MYB gene was down-regulated after insect infestation (Hui et al. 2003), while the *AtPAP1* gene, which encodes a MYB-like transcription factor, was implicated in the response to armyworm (*Spodoptera frugiperda*) (Johnson and Dowd 2004). The *Nicotiana tabacum* MYB gene *NtMYB1* has a role in plant resistance against *Tobacco mosaic virus* (Yang and Liu et al. 2004; Klessig 1996). Furthermore, expression of 15 *O. sativa* genes, nine *A. thaliana*, one *Nicotiana attenuata*, and one parsley WRKY gene was shown to be affected by fungal inoculation (Eulgem et al. 1999; McGrath et al. 2005; Ryu et al. 2006), insect infestation (Hui et al. 2003), and by chitin in our earlier DNA microarray experiments (Ramonell et al. 2005). Four *Arabidopsis* WRKY genes (*WRKY18*, *WRKY33*, *WRKY40*, and *WRKY60*) were found to be induced by the necrotrophic fungal pathogen *Botrytis cinerea* (Xu et al. 2006; Zheng et al. 2006). The *Arabidopsis WRKY11*, *WRKY17*, *WRKY18*, *WRKY40*, and *WRKY60* genes were induced by the bacterium *Pseudomonas syringae* (Journot-Catalino et al. 2006; Xu et al. 2006). Interestingly, out of these six WRKY genes, we identified five to be induced after 15 to 30 min of chitooctaose treatments (*WRKY33*, *WRKY11*, *WRKY17*, *WRKY18*, and *WRKY40*). Finally, the expression of two rice *GRAS* genes was shown to be affected by *N*-acetylchitooligosaccharide treatments (Day et al. 2003, 2004).

Recently, McGrath and associates (2005) also used high-throughput qRT-PCR screens to analyze the response of *Arabidopsis* to fungal pathogen inoculation (*Alternaria brassicicola*). A comparison of our results to this published study, reveals 18 TF genes responsive to fungus inoculation and chitin treatment, specifically, 10 AP2/ERE genes, two AthC2H2 zinc finger genes, two MYB genes, and four WRKY genes (Table 3). McGrath and associates (2005) also reported the results for

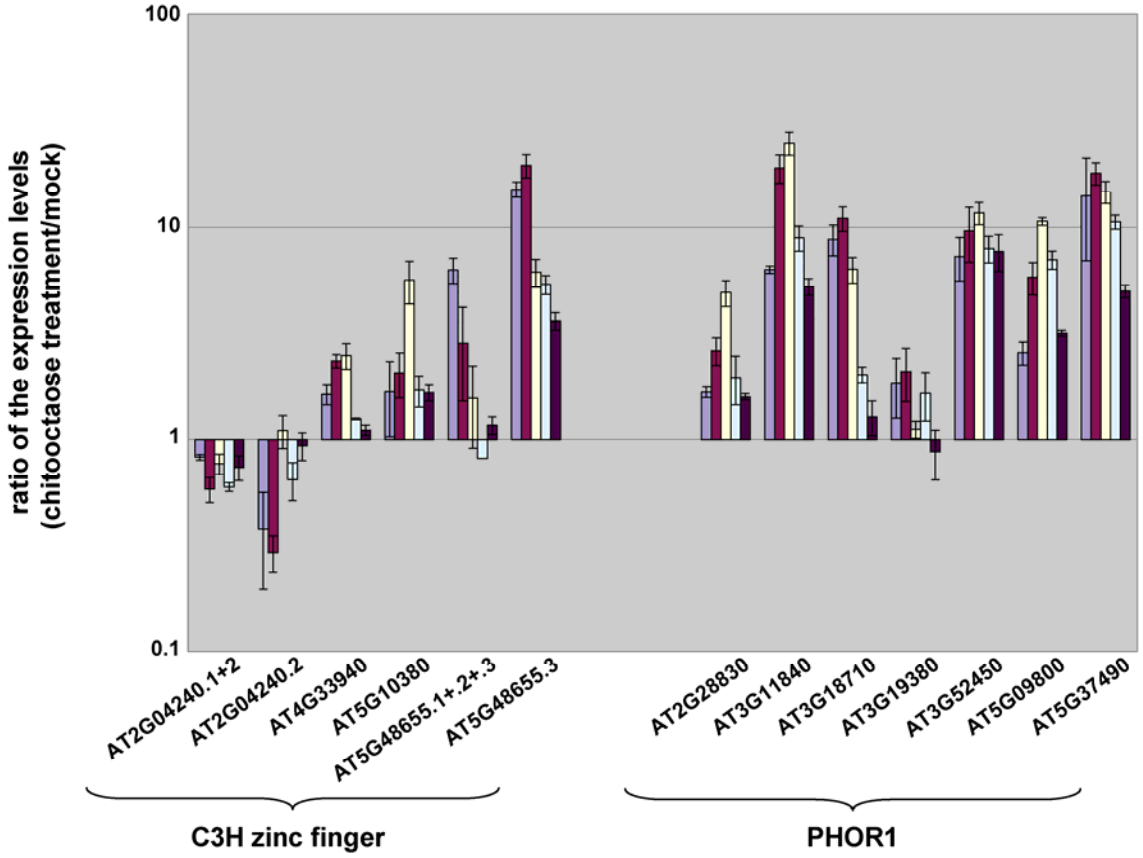

Fig. 3. Analysis of the expression levels of 11 *Arabidopsis thaliana* ubiquitin-ligase genes after chitooctaose treatment. Quantitative polymerase chain reactions were performed on cDNA isolated from plants treated with chitooctaose (left to right) 15 (blue), 30 (purple), 60 (yellow), 90 (blue light), and 120 (dark purple) min. The gene designations are shown on the *x* axis, while the *y* axis presents the ratio of the expression levels (chitooctaose treatment/mock).

TF gene expression in response to JA treatment. A comparison of these results to those of chitooctaose treatment identifies 15 TF genes (nine AP2/ERE, two AthC2H2 zinc finger, 1 MYB, and three WRKY genes) responsive to both treatments (Table 3). Collectively, a total of 20 TF genes were responsive to chitooctaose and to either JA treatment or fungal inoculation (Table 3). This type of comparison allows the identification of those TF genes most likely to play a crucial role in the integration of plant defense responses to biotic stress.

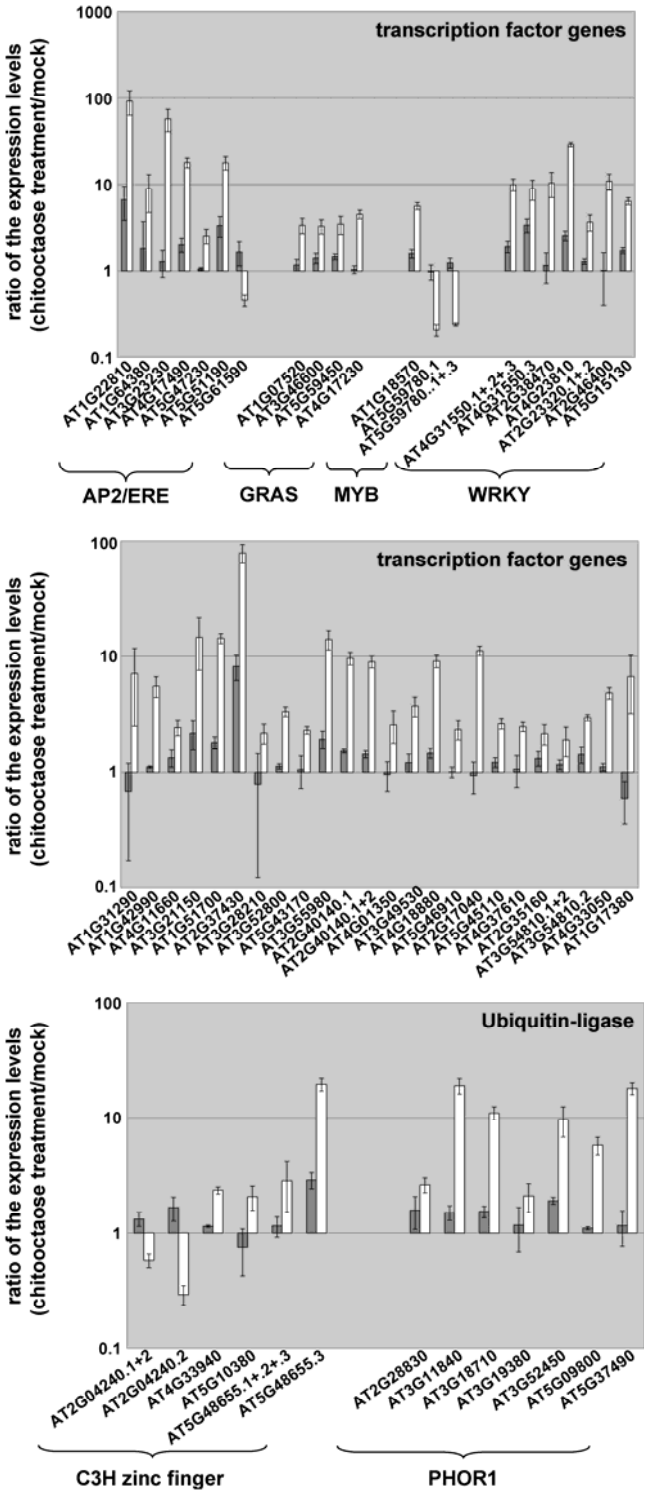

Fig. 4. Comparison of the expression levels of *Arabidopsis thaliana* ubiquitin-ligase and transcription factor genes after chitotetraose and chitooctaose treatment. Quantitative polymerase chain reactions were performed on cDNA isolated from plants treated 30 min with chitotetraose (gray) or chitooctaose (white) at a final concentration of 1 μM. The gene designations are shown on the *x* axis, while the *y* axis presents the ratio of the expression levels (chitin treatment/mock).

Flagellin, another PAMP elicitor, was shown to affect the expression of TF genes, specially the WRKY family (Navarro et al. 2004). More precisely, four WRKY genes (*AT4G31550*, *AT2G38470*, *AT4G23810*, and *AT2G23320*) were responsive to both chitooctaose and flagellin flg22. It is the interplay of such factors that likely governs the expression of specific genes.

Thirty ubiquitin-ligase like genes are regulated by chitin.

Our current study identified 30 putative ubiquitin-ligase-like genes whose expression responded to chitooctaose treatment (eight identified from the high-throughput qRT-PCR library and 22 identified by Affymetrix array with subsequent qRT-PCR confirmation with redesigned primers). Ubiquitin-ligases are involved in the ubiquitination of a target protein to target it for degradation. Ubiquitin-mediated protein degradation was previously shown to be involved in many developmental events and responses to environmental cues (Vierstra 2003). Increasing evidence also supports a critical role for ubiquitination and protein degradation in plant defense responses (Devoto et al. 2003; Vierstra 2003). The response of these genes to chitin elicitation is consistent with this idea.

Chitin differentially regulates transcript variants of some TF and ubiquitin-ligase genes.

Among the 118 TF genes and the 30 ubiquitin-ligase genes regulated by chitin, a few exhibit differential splicing resulting in different transcript variants. Chitooctaose treatment differentially affected the expression of transcript variants encoded by the *WRKY11* gene (*AT4G31550*) and two ubiquitin-ligase genes (*AT5G48655* and *AT2G04240*). Such differential responses by transcript variants are commonly observed in defense-related genes, e.g., the tobacco *N* gene (Dinesh-Kumar and Baker 2000) and *Mla* genes (Halterman et al. 2003). Recently, Li and associates (2006) discovered that the four tran-

Table 3. List of 20 transcription factor (TF) genes that were responsive to chitooctaose and to fungal or jasmonic acid treatment, or both[a]

Annmotation	Name	*Alternaria brassicicola*	MeJA
AP2/ERE gene family		•	
AT1G22810			
AT1G28370	*ATERF11/ERF11*	•	•
AT1G33760		•	•
AT1G75490			•
AT2G44840	*ATERF13*	•	•
AT3G15210	*ATERF-4/ATERF4/ERF4/RAP2.5*	•	•
AT3G23250		•	
AT3G50260			•
AT4G17490	*ATERF6*	•	
AT4G17500	*ATERF-1*	•	•
AT3G23230		•	•
AT5G47220	*ATERF-2/ATERF2/ERF2*	•	•
C2H2 zinc finger gene family			
AT1G27730	*ZAT10*	•	•
AT2G37430		•	•
MYB domain transcription factor gene family			
AT1G18570	*MYB51*	•	•
AT5G59780	*MYB27*	•	
WRKY domain transcription factor gene family			
AT1G80840	*WRKY40*	•	•
AT2G38470	*WRKY33*	•	•
AT4G23810	*WRKY53*	•	
AT4G31800	*WRKY18*	•	•

[a] Data from McGrath et al. 2005. • indicates response was present.

script variants encoded by an *Arabidopsis* MYB transcription factor gene (*AT5G59780*, *MYB59*) were differentially regulated by JA, SA, and ET, plant hormones important in mediating plant defense. The current data are consistent with the fact that cellular signaling involved in the plant defense response differentially affects the splicing of key regulators.

Chitin elicitation depends on *d.p.*

In general, the expression of the chitin-responsive genes responded specifically to the presence of chitooctaose (with a *d.p.* of 8) and was not responsive to treatment with chitotetraose (with a *d.p.* of 4). These results are in agreement with previous findings using *Arabidopsis* (Wan et al. 2004; Zhang et al. 2002) and a variety of other plant species (Cabrera et al. 2006; Shibuya and Minami 2001; Stacey and Shibuya 1997). However, smaller chitin fragments were shown to specifically activate ERK (extracellular regulated kinase) pathways in embryonic zebrafish cell lines (with a *d.p.* of 4; Snaar-Jagalska et al. 2003), to induce cortical cell divisions in red clover roots expressing the pea lectin gene (with a *d.p.* of 2 to 4; Diaz et al. 2000), and to induce extracellular alkalinization and protein phosphorylation in tomato cells (with a *d.p.* of 1 to 5; Felix et al. 1993, 1998). Therefore, further investigation may be warranted to explore whether plants possess pathways that respond to lower molecular weight chitin oligomers.

Real-time qPCR vs. DNA microarray.

Czechowski and associates (2004) in their original description of the use of qRT-PCR to screen the expression levels of *Arabidopsis* TF genes pointed to the >100-fold sensitivity of this method in comparison to DNA microarray hybridization. In the current study, we directly compared these two methods. Our results support the fact that the qRT-PCR method is more sensitive as evidenced by the identification of 33 additional chitin-responsive TF genes missed by DNA microarray analysis. However, there were also chitin-responsive genes that were found by DNA microarray hybridization that were missed in the high-throughput qRT-PCR screen. Some of these genes could be confirmed by qRT-PCR, using redesigned primers. Others may also reflect true expression and were simply missed by qRT-PCR due to suboptimal PCR primer design. Therefore, our results indicate that high-throughput qRT-PCR and DNA microarray hybridization methods are complementary approaches that, when used together, reveal the most complete picture of gene expression. These results also urge caution in blindly using qRT-PCR results with a single primer set as the sole confirming method for DNA microarray results.

MATERIALS AND METHODS

Plant culture conditions and chitin oligomer treatments.

Arabidopsis seeds were sterilized and grown hydroponically according to Zhang and associates (2002). Seedlings (14-day-old) were treated with either purified chitooctaose (Octa-N-acetylchitooctaose; Sigma, St. Louis) or chitotetraose (Tetra-N-Aceytlchitotetraose; Seikagaku Corporation, Tokyo) at a final concentration of 1 μM for the time indicated. The control seedlings were similarly treated with an equal amount of water.

RNA extraction, DNase treatments, and RT-PCR.

Total RNA was isolated from the collected seedlings using Trizol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) and subsequently purified using an RNeasy mini kit (Qiagen, Inc., Valencia, CA, U.S.A.) according to the manufacturers' instructions. Purified RNA (10 μg) was further treated with TURBO DNase (Ambion, Austin, TX, U.S.A.) to remove any contaminating genomic DNA, according to the manufacturer's instruction. Approximately 1.5 μg of DNA-free RNA was used for first-strand cDNA synthesis using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instruction. For use in the high-throughput qRT-PCR reactions, the RT reactions were performed with SuperScript III RT (Invitrogen). The cDNAs used for the kinetic analysis of the TF expression after chitin treatments were obtained after reverse transcription of 2 μg of RNA by M-MLV (Promega, Madison, WI, U.S.A.).

DNase treatment and cDNA synthesis quality were carefully monitored. The level of genomic DNA contamination was analyzed by qRT-PCR using primers designed to amplify the intron sequence of the AT5G65080 gene (5′-TTTTTTGCCCC CTTCGAATC-3′ and 5′-ATCTTCCGCCACCACATTGTAC-3′). To verify the quality of the RT reactions, qRT-PCR amplification with primers designed to amplify 5′ (5′-TCTCG ATCTCAATTTCGCAAAA-3′ and 5′-CGAAACCGTTGATT CCGATTC-3') and 3′ (5′-TTGGTGACAACAGGTCAAGCA-3′ and 5′-AAACTTGTCGCTCAATGCAATC-3′) regions of GADPH cDNA were performed. The 3' to 5' transcription ratio was >1.5 and <2 for all the samples analyzed.

qRT-PCR reaction conditions and data analysis.

The 2,297 primer sequences of the 2,227 TF genes and the primer sequences of the 34 TF genes and the nine ubiquitin-ligase genes analyzed by qPCR reactions are available in Supplemental Table VII. The high-throughput qRT-PCR screen was conducted at the Max Planck Institute of Molecular Plant Physiology, Gölm, Germany. The analysis of other genes, at different timepoints and in different conditions was performed at University of Missouri (Columbia, U.S.A.).

The qRT-PCR reactions were performed at Gölm in a 384-well plate format (7900 HT sequence detection system; Applied Biosystems, Foster City, CA, U.S.A.). The reaction setup was done on the robot Evolution P3 liquid handling system (Perkin Elmer, Wellesley, MA, U.S.A.). The qRT-PCR reactions were performed at the University of Missouri with a 96-well plate qRT-PCR machine (7500 real-time PCR system; Applied Biosystems). SYBR green PCR master mix (Applied Biosystems) was used to monitor cDNA synthesis. Primer sets (final concentration of 0.2 μM for each primer) were used for a final volume of 10 μl. The thermal profile of the qRT-PCR reactions was: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Moreover, to analyze the quality of the dissociation curves, the following program was added after the 40 PCR cycles: 95°C for 15 s, followed by a constant increase of the temperature from 60°C to 95°C. The data were analyzed with two different software packages. The qRT-PCR results were analyzed with SDS 2.2.1 software (Applied Biosystems). An Rn threshold of 0.2 was selected to obtain the cycle threshold (Ct) values (automatic background subtraction). PCR efficiencies (Peff) were calculated according to a linear regression analysis with LinRegPCR software (Ramakers et al. 2003). Only primer efficiencies were used to calculate the expression level of the genes if $R^2 > 0.995$ and Peff > 1.5.

The expression of the *AT2G28390* (SAND family protein) gene was used to normalize all the qRT-PCR results due to its highly stable expression (Czechowski et al. 2005). The *AT2G28390* Ct value was subtracted from the Ct values of the TF genes analyzed (ΔCt). Expression levels (Exp) of each TF gene were calculated according to the equation $\text{Exp} = \text{Peff}^{(-\Delta Ct)}$.

Three biological replicates for each treatment were analyzed. A Student *t*-test was used to compare statistically the gene expression after two different treatments. The expression level ratio between two conditions with *P* values <0.05 (95% confidence) are taken into consideration in this study.

Microarray experiment and data analysis.

Three biological replicates were analyzed for each condition. Total RNA (7μg), purified using an RNeasy mini kit (Qiagen Inc.), was used with the Affymetrix GeneChip expression 3′-amplification reagenes one-cycle cDNA synthesis system (Affymetrix, Santa Clara, CA, U.S.A.) to synthesize double-stranded cDNA according to the manufacturer's instructions. The synthesized cDNA was purified using the Affymetrix GeneChip sample cleanup module and was used with the Affymetrix GeneChip IVT labeling system to synthesize biotin-labeled cRNA. The purified biotin-labeled cRNA was fragmented according to the manufacturer's instructions. The arrays were hybridized, washed, and scanned, also according to the manufacturer's instructions.

The scanned data (CEL or DAT files) were analyzed using the dChip (DNA-Chip Analyzer) software (version release, April 1, 2005) (Li and Wong 2001a and b). The default settings were used in the normalization and model-based data analysis, with the following criteria selected for identifying significantly regulated genes: twofold, with a *t*-test *P* value <0.05, and the absolute signal intensity difference between a baseline and its corresponding treatment >100.

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LITERATURE CITED

Baureithel, K., Felix, G., and Boller, T. 1994. Specific, high affinity binding of chitin fragments to tomato cells and membranes. Competitive inhibition of binding by derivatives of chitooligosaccharides and a Nod factor of rhizobium. J. Biol. Chem. 269:17931-17938.

Bindschedler, L. V., Whitelegge, J. P., Millar, D. J., and Bolwell, G. P. 2006. A two component chitin-binding protein from French bean—Association of a proline-rich protein with a cysteine-rich polypeptide. FEBS (Fed. Eur. Biochem. Soc.) Lett. 580:1541-1546.

Boter, M., Ruiz-Rivero, O., Abdeen, A., and Prat, S. 2004. Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and *Arabidopsis*. Genes Dev. 18:1577-1591.

Cabrera, J. C., Messiaen, J., Cambier, P., and Van Cutsem, P. 2006. Size, acetylation and concentration of chitooligosaccharide elicitors determine the switch from defense involving PAL activation to cell death and water peroxide production in *Arabidopsis* cell suspensions. Physiol. Plant 127:44-56.

Czechowski, T., Bari, R. P., Stitt, M., Scheible, W. R., and Udvardi, M. K. 2004. Real-time RT-PCR profiling of over 1400 *Arabidopsis* transcription factors: Unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant J. 38:366-379.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., and Scheible, W. R. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 139:5-17.

Day, R. B., Okada, M., Ito, Y., Tsukada, K., Zaghouani, H., Shibuya, N., and Stacey, G. 2001. Binding site for chitin oligosaccharides in the soybean plasma membrane. Plant Physiol. 126:1162-1173.

Day, R. B., Shibuya, N., and Minami, E. 2003. Identification and characterization of two new members of the GRAS gene family in rice responsive to *N*-acetylchitooligosaccharide elicitor. Biochim. Biophys. Acta 1625:261-268.

Day, R. B., Tanabe, S., Koshioka, M., Mitsui, T., Itoh, H., Ueguchi-Tanaka, M., Matsuoka, M., Kaku, H., Shibuya, N., and Minami, E. 2004. Two rice GRAS family genes responsive to *N*-acetylchitooligosaccharide elicitor are induced by phytoactive gibberellins: Evidence for cross-talk between elicitor and gibberellin signaling in rice cells. Plant Mol. Biol. 54:261-272.

Desveaux, D., Marechal, A., and Brisson, N. 2005. Whirly transcription factors: Defense gene regulation and beyond. Trends Plant Sci. 10:95-102.

Devoto, A., Muskett, P. R., and Shirasu, K. 2003. Role of ubiquitination in the regulation of plant defence against pathogens. Curr. Opin. Plant Biol. 6:307-311.

Diaz, C. L., Spaink, H. P., and Kijne, J. W. 2000. Heterologous rhizobial lipochitin oligosaccharides and chitin oligomers induce cortical cell divisions in red clover roots, transformed with the pea lectin gene. Mol. Plant-Microbe Interact. 13:268-276.

Dinesh-Kumar, S. P., and Baker, B. J. 2000. Alternatively spliced N resistance gene transcripts: Their possible role in tobacco mosaic virus resistance. Proc. Natl. Acad. Sci. U.S.A. 97:1908–1913.

Eulgem, T. 2005. Regulation of the *Arabidopsis* defense transcriptome. Trends Plant Sci. 10:71-78.

Eulgem, T., Rushton, P. J., Schmelzer, E., Hahlbrock, K., and Somssich, I. E. 1999. Early nuclear events in plant defence signalling: Rapid gene activation by WRKY transcription factors. EMBO (Eur. Mol. Biol. Organ.) J. 18:4689-4699.

Eulgem, T., Rushton, P. J., Robatzek, S., and Somssich, I. E. 2000. The WRKY superfamily of plant transcription factors. Trends Plant Sci. 5:199-206.

Felix, G., Regenass, M., and Boller, T. 1993. Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: Induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state. Plant J. 4:307-316.

Felix, G., Baureithel, K., and Boller, T. 1998. Desensitization of the perception system for chitin fragments in tomato cells. Plant Physiol. 117:643-650.

Felix, G., Duran, J. D., Volko, S., and Boller, T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. 18:265-276.

Gomez-Gomez, L. 2004. Plant perception systems for pathogen recognition and defence. Mol. Immunol. 41:1055-1062.

Gutterson, N., and Reuber, T. L. 2004. Regulation of disease resistance pathways by AP2/ERF transcription factors. Curr. Opin. Plant Biol. 7:465-471.

Halterman, D. A., Wei, F., and Wise, R. P. 2003. Powdery mildew-induced Mla mRNAs are alternatively spliced and contain multiple upstream open reading frames. Plant Physiol. 131:558-567.

Heath, M. C. 2000. Nonhost resistance and nonspecific plant defenses. Curr. Opin. Plant Biol. 3:315-319.

Hui, D., Iqbal, J., Lehmann, K., Gase, K., Saluz, H. P., and Baldwin, I. T. 2003. Molecular interactions between the specialist herbivore *Manduca sexta* (lepidoptera, sphingidae) and its natural host *Nicotiana attenuata:* V. microarray analysis and further characterization of large-scale changes in herbivore-induced mRNAs. Plant Physiol. 131:1877-1893.

Johnson, E. T., and Dowd, P. F. 2004. Differentially enhanced insect resistance, at a cost, in *Arabidopsis* thaliana constitutively expressing a transcription factor of defensive metabolites. J. Agric. Food Chem. 52:5135-5145.

Journot-Catalino, N., Somssich, I. E., Roby, D., and Kroj, T. 2006. The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in *Arabidopsis thaliana.* Plant Cell 18:3289-3302.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proc. Natl. Acad. Sci. U.S.A. 18:11086-11091.

Kim, C. Y., Lee, S. H., Park, H. C., Bae, C. G., Cheong, Y. H., Choi, Y. J., Han, C., Lee, S. Y., Lim, C. O., and Cho, M. J. 2000. Identification of rice blast fungal elicitor-responsive genes by differential display analysis. Mol. Plant Microbe Interact. 13:470-474.

Kim, C. Y., and Zhang, S. 2004. Activation of a mitogen-activated protein kinase cascade induces WRKY family of transcription factors and defense genes in tobacco. Plant J. 38:142-151.

Li, C., and Wong, W. H. 2001a. Model-based analysis of oligonucleotide arrays: Expression index computation and outlier detection. Proc. Natl. Acad. Sci. U.S.A. 98:31-36.

Li, C., and Wong, W. H. 2001b. Model-based analysis of oligonucleotide arrays: Model validation, design issues and standard error application. Genome Biology 2: research0032.1-0032.11. Published online.

Li, J., Li, X., Guo, L., Lu, F., Feng, X., He, K., Wei, L., Chen, Z., Qu, L. J., and Gu, H. 2006. A subgroup of MYB transcription factor genes undergoes highly conserved alternative splicing in *Arabidopsis* and rice. J. Exp. Bot. 57:1263-1273.

Liu, Y., Schiff, M., Serino, G., Deng, X. W., and Dinesh-Kumar, S. P. 2002. Role of SCF ubiquitin-ligase and the COP9 signalosome in the N gene-mediated resistance response to *Tobacco mosaic virus*. Plant Cell 14:1483-1496.

Liu, Y., Schiff, M., and Dinesh-Kumar, S. P. 2004. Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, COI1 and

CTR1 in *N*-mediated resistance to tobacco mosaic virus. Plant J. 38:800-809

Liu, X. Q., Bai, X. Q., Qian, Q., Wang, X. J., Chen, M. S., and Chu, C. C. 2005. OsWRKY03, a rice transcriptional activator that functions in defense signaling pathway upstream of OsNPR1. Cell Res. 15:593-603.

Lorenzo, O., Chico, J. M., Sanchez-Serrano, J. J., and Solano, R. 2004. JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. Plant Cell 16:1938-1950.

McGrath, K. C., Dombrecht, B., Manners, J. M., Schenk, P. M., Edgar, C. I., Maclean, D. J., Scheible, W. R., Udvardi, M. K., and Kazan, K. 2005. Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified *via* a genome-wide screen of *Arabidopsis* transcription factor gene expression. Plant Physiol 139:949-959.

Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., Boller, T., and Jones, J. D. 2004. The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. Plant Physiol. 135:1113-1128.

Nürnberger, T., Brunner, F., Kemmerling, B., and Piater, L. 2004. Innate immunity in plants and animals: Striking similarities and obvious differences. Immunol. Rev. 198:249-266.

Park, C. J., Shin, Y. C., Lee, B. J., Kim, K. J., Kim, J. K., and Paek, K. H. 2006. A hot pepper gene encoding WRKY transcription factor is induced during hypersensitive response to *Tobacco mosaic virus* and *Xanthomonas campestris*. Planta 223:168-179.

Ramakers, C., Ruijte, J. M., Deprez, R. H., and Moorman, A. F. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339:62-66.

Ramonell, K. M., Zhang, B., Ewing, R. M., Chen, Y., Xu, D., Stacey, G., and Somerville, S. 2002. Microarray analysis of chitin elicitation in *Arabidopsis* thaliana. Mol. Plant Pathol. 3:301-311.

Ramonell, K., Berrocal-Lobo, M., Koh, S., Wan, J., Edwards, H., Stacey, G., and Somerville, S. 2005. Loss-of-function mutations in chitin responsive genes show increased susceptibility to the powdery mildew pathogen *Erysiphe cichoracearum*. Plant Physiol 138:1027-1036.

Ryu, H. S., Han, M., Lee, S. K., Cho, J. I., Ryoo, N., Heu, S., Lee, Y. H., Bhoo, S. H., Wang, G. L., Hahn, T. R., and Jeon, J. S. 2006. A comprehensive expression analysis of the WRKY gene superfamily in rice plants during defense response. Plant Cell Rep. 25:836-47

Schmelz, E. A., Alborn, H. T., and Tumlinson, J. H. 2003. Synergistic interactions between volicitin, jasmonic acid and ethylene mediate insect-induced volatile emission in *Zea mays*. Physiol. Plant 117:403-412.

Shibuya, N., and Minami, E. 2001. Oligosaccharide signaling for defense responses in plant. Physiol. Mol. Plant Pathol. 59:223-233.

Snaar-Jagalska, B. E., Krens, S. F. G., Robina, I., Wang, L. X., and Spaink, H. P. 2003. Specific activatin of ERK pathways by chitin oligosaccharides in embryonic zebrafish cell lines. Glycobiology 13:725-732.

Stacey, G., and Shibuya, N. 1997. Chitin recognition in rice and legumes. Plant Soil 194:161-169.

Stacey, G., Libault, M., Brechenmacher, L., Wan, J., and May, G. D. 2006. Genetics and functional genomics of legume nodulation. Curr. Opin. Plant Biol. 9:110-121.

Tanabe, S., Okada, M., Jikumaru, Y., Yamane, H., Kaku, H., Shibuya, N., Minami, E. 2006. Induction of resistance against rice blast fungus in rice plants treated with a potent elicitor, *N*-acetylchitooligosaccharide. Biosci. Biotechnol. Biochem. 70(7):1599-1605.

Thurow, C., Schiermeyer, A., Krawczyk, S., Butterbrodt, T., Nickolov, K., and Gatz, C. 2005. Tobacco bZIP transcription factor TGA2.2 and related factor TGA2.1 have distinct roles in plant defense responses and plant development. Plant J. 44:100-113.

Vailleau, F., Daniel, X., Tronchet, M., Montillet, J. L., Triantaphylides, C., and Roby, D. 2002. A R2R3-MYB gene, *AtMYB30*, acts as a positive regulator of the hypersensitive cell death program in plants in response to pathogen attack. Proc. Natl. Acad. Sci. U.S.A. 99:10179-10184.

Vierstra, R. D. 2003. The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. Trends Plant Sci. 8:135-142.

Wan, J., Zhang, S., and Stacey, G. 2004. Activation of a mitogen-activated protein kinase pathway in *Arabidopsis* by chitin. Mol. Plant Pathol. 5:125-135.

Xu, X., Chen, C., Fan, B., and Chen, Z. 2006. Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell. 18:1310-1326.

Yanagisawa, S. 2002. The Dof family of plant transcription factors. Trends Plant Sci. 7:555-560.

Yang, Y., and Klessig, D. F. 1996. Isolation and characterization of a *Tobacco mosaic virus*-inducible myb oncogene homolog from tobacco. Proc. Natl. Acad. Sci. U.S.A. 93:14972-14977.

Yang, C. W., Gonzalez-Lamothe, R., Ewan, R. A., Rowland, O., Yoshioka, H., Shenton, M., Ye, H., O'Donnell, E., Jones, J. D., and Sadanandom, A. 2006. The E3 ubiquitin ligase activity of *Arabidopsis* plant U-BOX17 and its functional tobacco homolog ACRE276 are required for cell death and defense. Plant Cell 18:1084-1098.

Zhang, B., Ramonell, K., Somerville, S., and Stacey, G. 2002. Characterization of early, chitin-induced gene expression in *Arabidopsis*. Mol. Plant Microbe Interact. 15:963-970.

Zheng, Z., Qamar, S. A., Chen, Z., and Mengiste, T. 2006. Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J. 48:592-605.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D., Felix, G., and Boller, T. 2004. Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature 428:764-776.

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