the plant journal

SEB Society for Experimental Biology

The Plant Journal (2012) doi: 10.1111/j.1365-313X.2011.04891.x

Volatiles of two growth-inhibiting rhizobacteria commonly engage AtWRKY18 function

Katrin Wenke[1], Dierk Wanke[2], Joachim Kilian[2], Kenneth Berendzen[2], Klaus Harter[2] and Birgit Piechulla[1,*]

[1]*Institute of Biological Sciences, Biochemistry, University of Rostock, Albert Einstein Straße 3, D-18059 Rostock, Germany, and*
[2]*Center for Plant Molecular Biology, University Tübingen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany*

Received 8 November 2011; accepted 7 December 2011.
*For correspondence (fax +49 381 498 6132; e-mail birgit.piechulla@uni-rostock.de).

SUMMARY

Interactions with the (a)biotic environment play key roles in a plant's fitness and vitality. In addition to direct surface-to-surface contact, volatile chemicals can also affect the physiology of organism. Volatiles of *Serratia plymuthica* and *Stenotrophomonas maltophilia* significantly inhibited growth and induced H_2O_2 production in Arabidopsis in dual culture. Within 1 day, transcriptional changes were observed by promoter–GUS assays using a stress-inducible W-box-containing 4x*GST1* construct. Expression studies performed at 6, 12 and 24 h revealed altered transcript levels for 889 genes and 655 genes in response to *Se. plymuthica* or *St. maltophilia* volatiles, respectively. Expression of 162 genes was altered in both treatments. Meta-analysis revealed that specifically volatile-responsive genes were significantly overlapping with those affected by abiotic stress. We use the term mVAMP (microbial volatile-associated molecular pattern) to describe these volatile-specific responses. Genes responsive to both treatments were enriched for W-box motifs in their promoters, and were significantly enriched for transcription factors (*ERF2*, *ZAT10*, *MYB73* and *WRKY18*). The susceptibility of *wrky18* mutant lines to volatiles was significantly delayed, suggesting an indispensable role for WRKY18 in bacterial volatile responses.

Keywords: *Arabidopsis thaliana*, Serratia plymuthica, WRKY, volatile organic compounds, microbe plant interaction, Stenotrophomonas maltophilia, microarray analysis, biotic stress, abiotic stress.

INTRODUCTION

Plants have to cope with various (a)biotic stresses, both aboveground and underground. The soil close to plant roots (rhizosphere) is highly attractive for a vast number of organisms because of the presence of carbon-rich root exudates (Barber and Martin, 1976). Consequently, plants have to interact in a complex manner with soil-dwelling organisms (e.g. nematodes, amoeba and bacteria). Diverse elicitors are used for multiple communications underground. The phenomenon of volatile-dependent interaction is still not very well understood, but some studies support a role for volatile signals emitted by plant roots, soil fungi and rhizobacteria (reviewed by Wenke *et al.*, 2010). Bacteria in particular are a rich source of various volatile compounds (Schulz and Dickschat, 2007). As volatile synthesis consumes energy and carbon, interesting questions arise regarding possible functions of airborne compounds. In 1972, Stall *et al.* demonstrated that ammonia produced by *Xanthomonas vesicatoria* played a role in necrosis formation in inoculated pepper (Stall *et al.*, 1972), and bacterial cyanide and ethylene have been shown to influence plant growth and physiology negatively (Alström and Burns, 1989; Weingart and Völksch, 1997; Fukuda *et al.*, 1993). Butanediol and acetoin emitted by plant growth-promoting rhizobacteria were shown to be sufficient for positive growth effects on Arabidopsis (Ryu *et al.*, 2003). The antagonistic potential of bacterial volatiles against the pathogenic fungus *Rhizoctonia solani* was demonstrated for various rhizobacteria, with *Serratia plymuthica* HRO-C48 and *Stenotrophomonas maltophilia* R3089 being the most effective (Kai *et al.*, 2007). This inhibition of plant pathogens by volatiles may lead to indirect promotion of plant fitness. In contrast, dramatic growth-inhibitory effects of airborne microbial metabolites were observed for *Arabidopsis thaliana* and *Physcomitrella patens* (Kai *et al.*, 2009; Vespermann *et al.*, 2007; Kai and Piechulla, 2010).

Se. plymuthica HRO-C48 and *St. maltophilia* R3089 volatiles also have growth-inhibitory effects on *A. thaliana*. The initial phase of plant responses triggered by volatiles of these bacteria are not understood. Appropriate signal processing is indispensable for fine-tuned modulation of

inputs to achieve a specific output (Dietz *et al.*, 2010). *In planta*, this fine-tuning is achieved by complex protein interaction in extensive signaling networks such as WRKY dependent signaling (Euglem and Somssich, 2007). In Arabidopsis, 74 members of the *WRKY* family have been identified (Eulgem *et al.*, 2000; Dong *et al.*, 2003). Several appear to play pivotal roles in regulating plant disease resistance by amplification and modulation of pathogen-induced signals, leading to specific and efficient responses. In order to obtain insight into transduction networks activated by bacterial volatiles, we performed microarray analysis of *A. thaliana* co-cultivated with *Se. plymuthica* HRO-C48 and *St. maltophilia* R3089.

RESULTS

Growth inhibition of Arabidopsis by rhizobacterial volatiles

We used split-dish plates for co-cultivation of seedlings with *Se. plymuthica* HRO-C48 or *St. maltophilia* R3089 (Kai *et al.*, 2007, 2008) (Figure 1a). Visual inspection of these seedlings revealed obvious phenotypic alterations compared to non-treated controls: within a few days, volatiles caused significantly decreased growth of cotyledons and primary roots (Figure 1b–d) as well as leaf yellowing (Figure 1b). To quantify chlorosis, we measured chlorophyll and carotenoid content (Figure 1e,f) and found a significant loss of total chlorophyll after 3 days of co-cultivation with *Se. plymuthica* (Figure 1e), while carotenoid content significantly decreased within 2 days of co-cultivation. *St. maltophilia* volatiles also affected chlorophyll and carotenoid contents but with a delayed response. Chlorophyll and carotenoid contents decreased significantly about 1 day after visible growth inhibition. To rule out the possibility that the observed differences originated from differing bacterial growth, we plated defined titers of bacteria on plates (4 and 1×10^7 colony-forming units of *Se. plymuthica* and *St. maltophilia*, respectively) and analyzed their growth. At the start of co-cultivation, *Se. plymuthica* grew

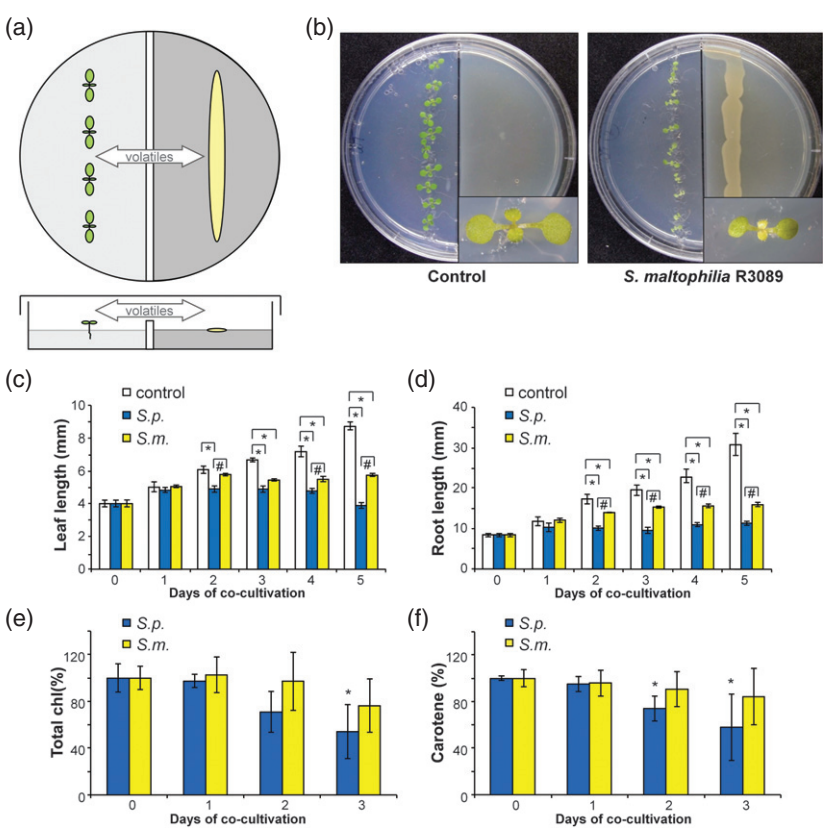

Figure 1. Phenotype of wild-type plants in dual-culture.
(a) Schematic top and side views of dual-culture of *A. thaliana* and rhizobacteria. The arrow indicates free exchange of volatiles above the plastic barrier between seedlings and bacteria. The barrier separates the different media in the two compartments.
(b) Representative example of co-cultivation of *A. thaliana* with *St. maltophilia* compared to control.
(c) Leaf length of cotyledons of control plants and seedlings co-cultivated with *Se. plymuthica* ($n = 6$) and *St. maltophilia* ($n = 3$).
(d) Length of primary roots of seedlings in dual-culture with *Se. plymuthica* ($n = 6$) and *St. maltophilia* ($n = 3$) or untreated.
(e,f) Relative chlorophyll *a* and *b* and carotenoid contents in seedlings co-cultivated with *Se. plymuthica* or *St. maltophilia* compared to untreated controls (100%) ($n = 6$).
Asterisks and hash symbols in (c–f) indicate $P \leq 0.01$ (paired Student's *t* test).

little faster than *St. maltophilia*. However, after 24 h of co-cultivation, both bacterial strains displayed equivalent cell numbers (Figure S1).

Volatile-induced H_2O_2 synthesis and promoter activities

H_2O_2 functions in many, if not all, stress responses as a rather non-specific second messenger (Neill *et al.*, 2002). Therefore, we examined volatile-induced H_2O_2 accumulation using diaminobenzidine (Thordal-Christensen *et al.*, 1997). After 48 h of co-cultivation with *Se. plymuthica*, a long-lasting presence of H_2O_2 was detected (Figure 2a). In accordance with previous findings on growth inhibition, we observed a delay of 1 day for H_2O_2 accumulation in response to *St. maltophilia* volatiles (Figure 2a).

To further dissect possible signaling cascades, we used *A. thaliana* lines expressing the *uidA* (GUS) gene controlled by synthetic plant promoters containing defined stress-responsive *cis*-regulatory DNA elements (Rushton *et al.*, 2002). Use of 4x*GST1* reproducibly resulted in strong GUS activation within 18 h of co-cultivation for both of the treatments (Figure 2b).

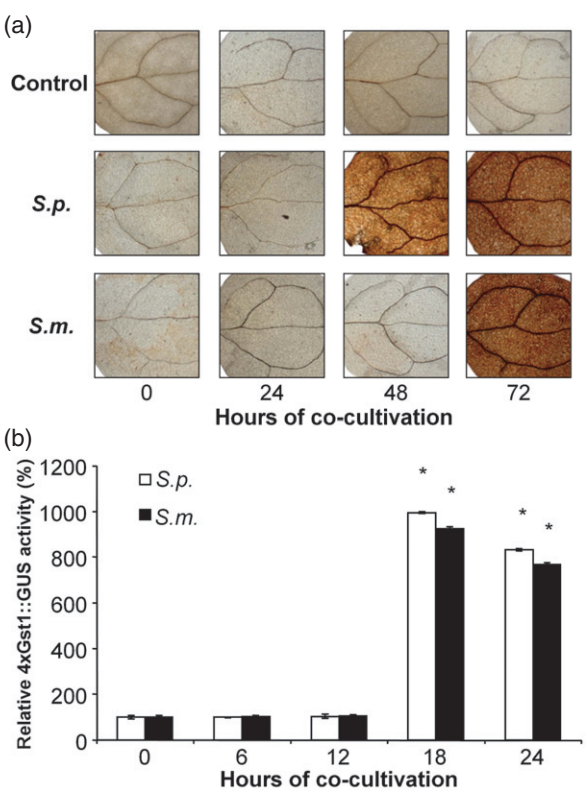

Figure 2. Physiological responses in *A. thaliana* seedlings in dual-culture.
(a) Diaminobenzidine staining of cotyledons after co-cultivation with *Serratia plymuthica* or *Stenotrophomonas maltophilia* compared to controls. H_2O_2 reacts with diaminobenzidine to form a brownish precipitate.
(b) Relative GUS reporter activity of *4xGST1::GUS* seedlings in response to *Se. plymuthica* and *St. maltophilia* volatiles compared to control plants (100%). The GUS reporter assay was performed using 4-methyl umbelliferyl glucuronide (MUG). Asterisks indicate $P \leq 0.001$ for comparison with time zero (Student's *t* test, $n = 3$).

Specific gene expression changes in response to rhizobacterial volatiles

To obtain a comprehensive understanding of underlying signaling processes, we performed a transcriptome analysis for *Se. plymuthica* and *St. maltophilia* volatile-triggered responses at 6, 12 and 24 h of co-cultivation. We used the ATH1 GeneChip array (Hennig *et al.*, 2003) and monitored transcript levels for two biological replicates for both rhizobacteria independently. Plant material was pooled from several plates for each replicate. The data were verified by quantitative RT-PCR using selected marker genes. A total of 889 and 655 genes were regulated in response to *Se. plymuthica* and *St. maltophilia* volatiles, respectively (Figure 3a,b; Tables S1, S2 and S3). In both of the treatments, more genes were repressed compared to the untreated control plants, but with different kinetics: *Se. plymuthica* volatiles induced major changes at 24 h of co-cultivation (Figure 3a), but the highest number of responsive genes was detected after 6 h of co-cultivation with *St. maltophilia* (Figure 3b). Only 162 genes were commonly responsive to the volatile profiles emitted by the two bacterial species (Table S1): these may contain signaling elements that cause plant growth inhibition and chlorosis in response to both rhizobacteria (Figure 3c and Figure S2).

To enable easier data visualization and comparison, we next processed volatile-responsive genes using MapMan (Thimm *et al.*, 2004). The expression patterns for *Se. plymuthica* volatiles were characteristic of expression patterns of known responses to pathogen attack (Figure 3d,e): up-regulated genes included At5g45070 and At1g65390, which encode two TIR-NBS-LRR-like receptors, and down-regulated genes included genes encoding peroxidases or glutathione-*S*-transferases or products involved in secondary metabolism or hormone signaling (salicylic acid, abscisic acid and auxin). Activated signal pathways involve ethylene synthesis and the corresponding ERF class of transcription factors (Figure 3d). There is also involvement of some pathogenesis-related (PR) genes, including seven TIR-NBS-LRR class proteins. *St. maltophilia* volatiles did not induce regulation of biotic stress-related genes other than some PR genes, e.g. four TIR-NBS-LRR class proteins (Figure 3e). Nonetheless, both responses involve alteration of cell-wall metabolism and proteolysis, and induction of members of the ERF, bZIP, MYB and WRKY transcription factor families (Figure 3d,e).

The 'metabolism overview' underlined the importance of processes at the cell wall (Figure S3). Cell wall-modifying enzymes are regulated in response to both rhizobacteria, and more were up-regulated in response to *St. maltophilia* volatiles. *St. maltophilia* volatiles led to repression of genes

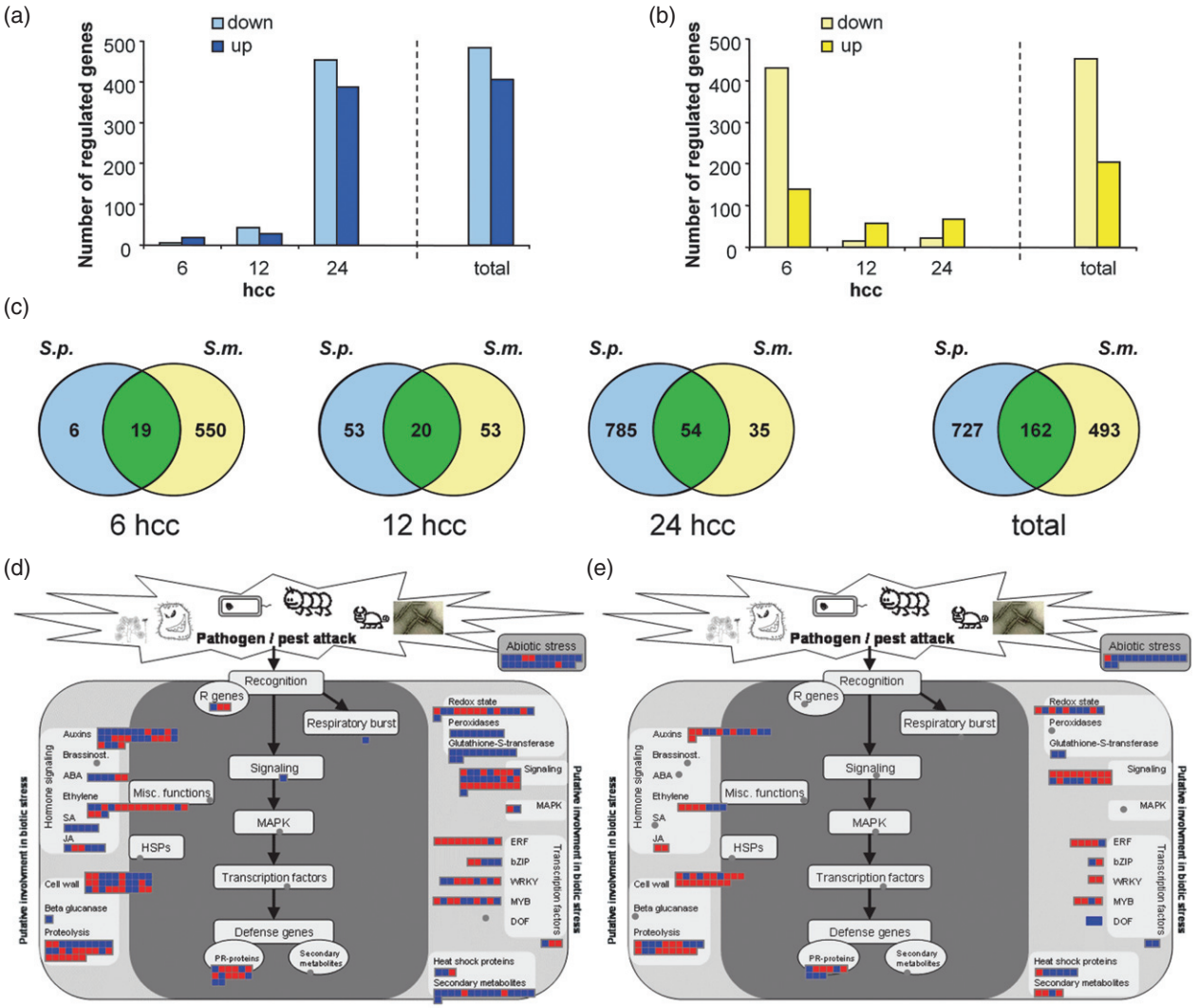

Figure 3. Volatile-induced gene expression response during co-cultivation.
(a,b) Total number of genes down- or up-regulated in wild-type seedlings in response to *Se. plymuthica* (a) or *St. maltophilia* (b) volatiles. The bacterial titer was 10^7 colony-forming units at the onset of the experiment.
(c) Expression changes after exposure to *Se. plymuthica* and *St. maltophilia* volatiles. The Venn diagrams depict specific and intersecting responses between the two rhizobacteria. The total number of responsive genes at 6, 12 and 24 h of dual-culture, and over all time points are shown.
(d,e) Genes responsive to *Se. plymuthica* (d) and *St. maltophilia* (e) volatiles are visualized using the 'biotic stress overview' in MapMan. Induced (red) or repressed (blue) genes with at least a twofold difference from controls are represented by individual squares.

encoding components of the mitochondrial electron transport chain and photosystems.

Functional classification of volatile-responsive genes

To determine whether differentially expressed genes correlated with functional activities, they were functionally classified using gene ontology (GO) terms at the Arabidopsis Information Resource (Swarbreck *et al.*, 2008; Berardini *et al.*, 2004). Expression of genes in the categories 'biological process', 'cellular component' and 'molecular function' is shown in Figure 4(a–c). Functional categorization was performed independently for the 727 and 493 specific genes and the 162 commonly regulated genes (Tables S1–S3).

The *Se. plymuthica*-specific genes were significantly depleted in GO terms associated with 'DNA, RNA and protein metabolism' and 'cell organization or biogenesis', but were enriched in 'transcription' and 'general responses to stress' (Figure 4a). Consistently, more genes had transcription factor (TF) activity than expected (Figure 4c). In contrast, genes encoding proteins with function in 'extracellular processes' were significantly enriched, while responses in the categories 'mitochondria', 'cytoplasm' and 'plasma membrane' appear to be depleted (Figure 4b).

Interestingly, in respect of GO terms the genes that responded specifically upon *St. maltophilia* volatiles exhibited only little overlap with *Se. plymuthica* volatile

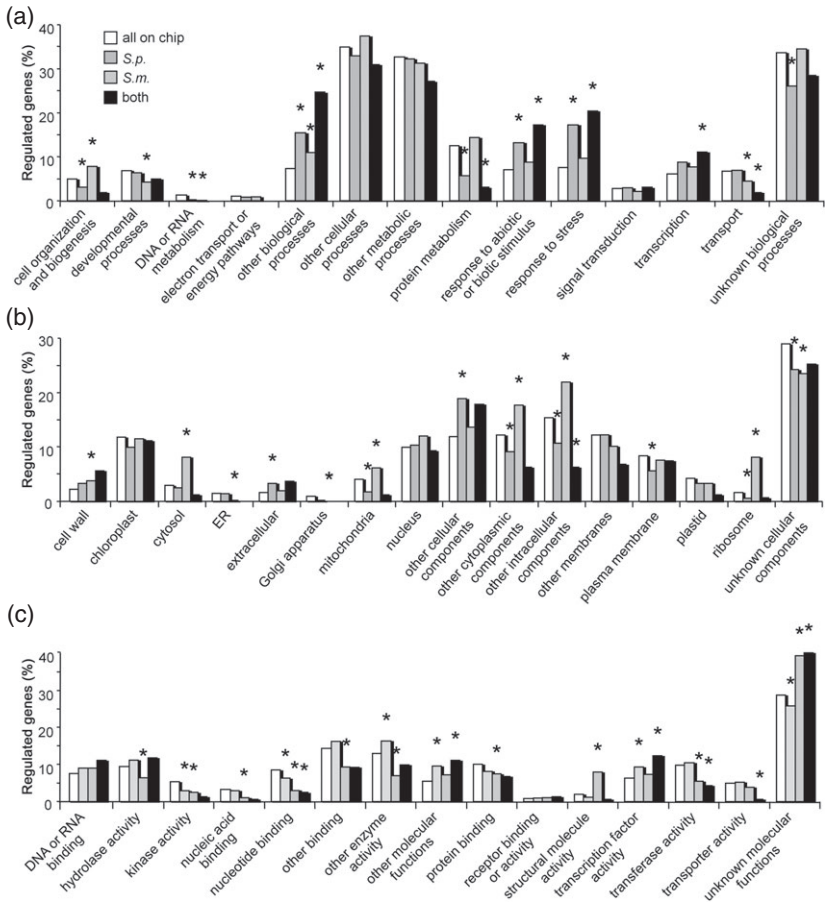

Figure 4. Functional categorization of volatile-responsive genes.
Number of genes for each particular gene ontology (GO) category as a percentage for all probe sets on the ATH1 chip, all responsive genes specific for *Serratia plymuthica* or *Stenotrophomonas maltophilia*, and the subset of genes that was involved in both responses. Categorization was performed with the web interface at The Arabidopsis Information Resource TAIR (Berardini *et al.*, 2004) using the keyword categories 'biological process' (a), 'cellular component' (b) and 'molecular function' (c). Asterisks indicate significant over- or under-representation of functional categories ($P \leq 0.005$).

responses. The *St. maltophilia* volatile-responsive genes were significantly enriched in functions for 'cell organization or biogenesis' and processes located inside the 'mitochondria' or 'cytosol', while *Se. plymuthica* volatile-responsive genes were depleted in these processes (Figure 4). Fewer genes responding to *St. maltophilia* volatiles were linked to 'transport', 'kinase activity', 'nucleic acid binding', 'transferase activity' and 'developmental processes'.

The 162 commonly responsive genes were enriched for general 'responses to abiotic and biotic stress' and 'transcription factor activity', but were depleted for 'transport activity' and 'protein metabolism' (Figure 4). Indeed, 21 TF genes were contained in the dataset, which is significantly more ($P \leq 1.3 \times 10^{-42}$) than expected (Table 1). Several of these genes have functions in developmental processes, e.g. BT2 and BT4 (BTB domain scaffold proteins; Robert *et al.*, 2009), or in stress responses, e.g. C_2H_2-type ZAT10 family protein or WRKY18 (Sakamoto *et al.*, 2000; Rossel *et al.*, 2007; Wang *et al.*, 2008).

Meta-data analysis

To obtain a better insight into the nature of plant's response to bacterial co-cultivation, we compared our datasets with publically available microarray datasets of (a)biotic stress responses, hormone treatments and response to growth-promoting *Bacillus subtilis* volatiles (Figure 5) (Kilian *et al.*, 2007; Goda *et al.*, 2008; Wanke *et al.*, 2009; Zhang *et al.*, 2007). Specifically and commonly regulated genes (Tables S1–S3) were matched independently against reference genes. To determine significance for pairwise comparisons, we calculated *P* (hypergeometric probability) for intersecting sets (Figure 5b).

To our surprise, the datasets for stress, hormone and volatiles followed similar trends. In accordance with the MapMan results (Figure 3d,e), there is only little overlap between volatile and biotic stress responses (Figure 5). Only some *Se. plymuthica*-specific genes were involved in responses to biotic interactions and gene-for-gene resis-

Table 1 Transcription factor genes that are responsive to *Se. plymuthica* HRO-C48 and *St. maltophilia* R3089 volatiles

AGI	Description
At1g21910	AP2 domain-containing transcription factor family protein
At5g61590	AP2 domain-containing transcription factor family protein
At2g44940	AP2 domain-containing transcription factor TINY
At1g13260	DNA-binding protein RAV1
At5g47220	Ethylene-responsive element-binding factor 2 (ERF2)
At3g24500	Ethylene-responsive transcriptional co-activator, putative
At3g61890	Homeobox-leucine zipper protein 12 (HB-12)/HD-ZIP12
At2g44910	Homeobox-leucine zipper protein 4 (HB-4)/HD-ZIP4
At1g01520	Myb family transcription factor
At3g50060	Myb family transcription factor
At2g23290	Myb family transcription factor
At1g18330	Myb family transcription factor
At4g37260	Myb family transcription factor (MYB73)
At1g56650	Myb family transcription factor (MYB75)
At2g02710	PAC motif-containing protein
At3g48360	Speckle-type POZ protein-related (BT2)
At5g67480	TAZ zinc finger family protein/BTB/POZ domain-containing protein (BT4)
At4g31800	WRKY family transcription factor (WRKY18)
At3g21890	Zinc finger (B-box type) family protein
At1g27730	Zinc finger (C_2H_2 type) family protein (ZAT10)
At3g55980	Zinc finger (CCCH-type) family protein

tance. Similarly, other than abscisic acid (ABA) and methyl jasmonate responses, there was no significant overlap between hormone-responsive genes and volatile-responsive genes. In contrast, a significant portion of genes overlapped with abiotic stress responses (Kilian *et al.*, 2007; Wanke *et al.*, 2009), for which hypergeometric probabilities were highest (Figure 5b). Many genes involved in response to cold, osmotic or salt stress and UV-B light were also differentially regulated in volatile datasets. Only a few genes that respond to *Bacillus subtilis* volatiles were also affected by *Se. plymuthica* or *St. maltophilia* volatiles.

Analysis of *cis*-regulatory promoter elements

The 162 commonly responding genes contain a high number of genes encoding TFs (Table 1). Therefore, analysis of already known functional *cis*-regulatory elements was performed using Athena (O'Connor *et al.*, 2005). Only 12 TF binding motifs were significantly enriched ($P \leq 10^{-3}$) (Table 2). Other than the TATA box motif, all other elements are known to regulate stress-responsive genes, mostly involved in adaptation to (a)biotic signals, ABA or light responses. Interestingly, the W box consensus TTGACY was present in 124 of the 162 promoters (Table 2).

Response of *WRKY* mutants to rhizobacterial volatiles

Significant enrichment of W boxes in the promoters of the 162 commonly responding genes and its presence in the 4x*GST1* element suggested a possible role for WRKYs in responses to volatiles. However, *AtWRKY18* (At4g31800) was the only gene that was significantly induced in both co-cultivation experiments (Figure 6c). AtWRKY18 is a member of the group IIa WRKY proteins and act partially

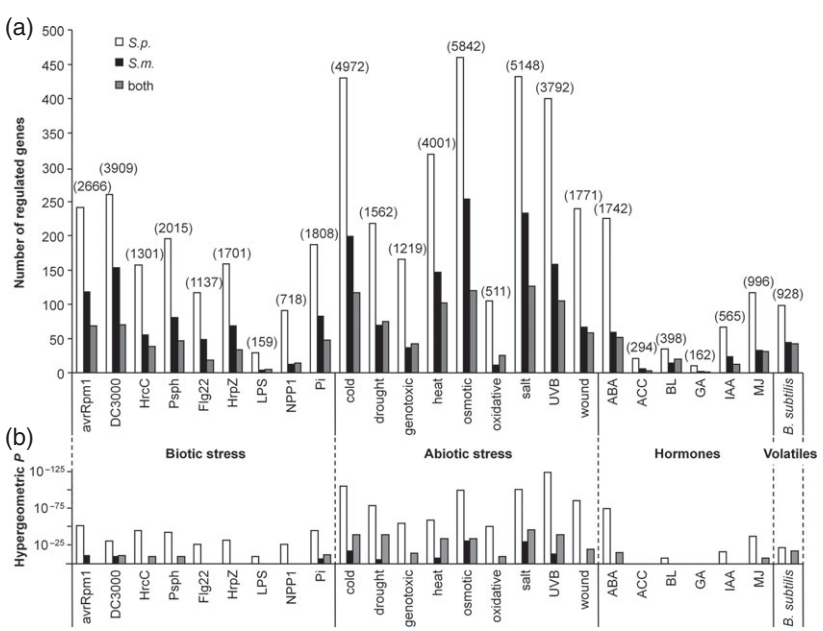

Figure 5. Meta-data analysis.
(a) The number of genes regulated specifically in response to *Serratia plymuthica* or *Stenotrophomonas maltophilia* volatiles or both were compared with datasets for biotic or abiotic stress-, hormone- or volatile-responsive genes. The total number of genes contained in datasets is given in parentheses above columns.
(b) Hypergeometric probabilities as a measure of significant overlap.

Table 2 Significantly enriched *cis*-regulatory elements in the promoters of the 162 commonly responsive genes

Motif	Description	*P*	Number of promoters with TF sites	Number of TF sites
TATAAA	TATA-box motif (promoter element at approximately -30 from transcription start)	$<10^{-5}$	147	684
WAACCA	MYB1AT (stress response)	$<10^{-3}$	142	424
CWWWWWWWWG	CARGCW8GAT (light and circadian cycle motif)	$<10^{-5}$	125	540
TTGACY	W-box promoter motif (WRKY class binding site, senescence and plant defense)	$<10^{-3}$	124	278
GATAAG	Ibox promoter motif (light-responsive element)	$<10^{-4}$	87	144
CATGTG	MYCATERD1 (water stress)	$<10^{-5}$	87	148
CACATG	AtMYC2 binding site in RD22 (transcriptional activators in abscisic acid signaling)	$<10^{-5}$	87	148
BACGTGKM	ABRE-like binding site motif (ABA regulation)	$<10^{-6}$	63	110
RCCGAC	DRE core motif (dehydration- and low-temperature stress-inducible)	$<10^{-3}$	55	77
CACGTG	CACGTGMOTIF (light)	$<10^{-3}$	44	118
RCCGACNT	DREB1A/CBF3 (cold stress response)	$<10^{-4}$	28	32
AAAATATCT	EveningElement promoter motif (circadian control)	$<10^{-3}$	27	37

The number of promoters containing the DNA motif consensus and the total number of transcription factor binding sites are given. The hypergeometric probability *P* is provided for the -1500 bp sequence upstream of the translation start site (ATG).

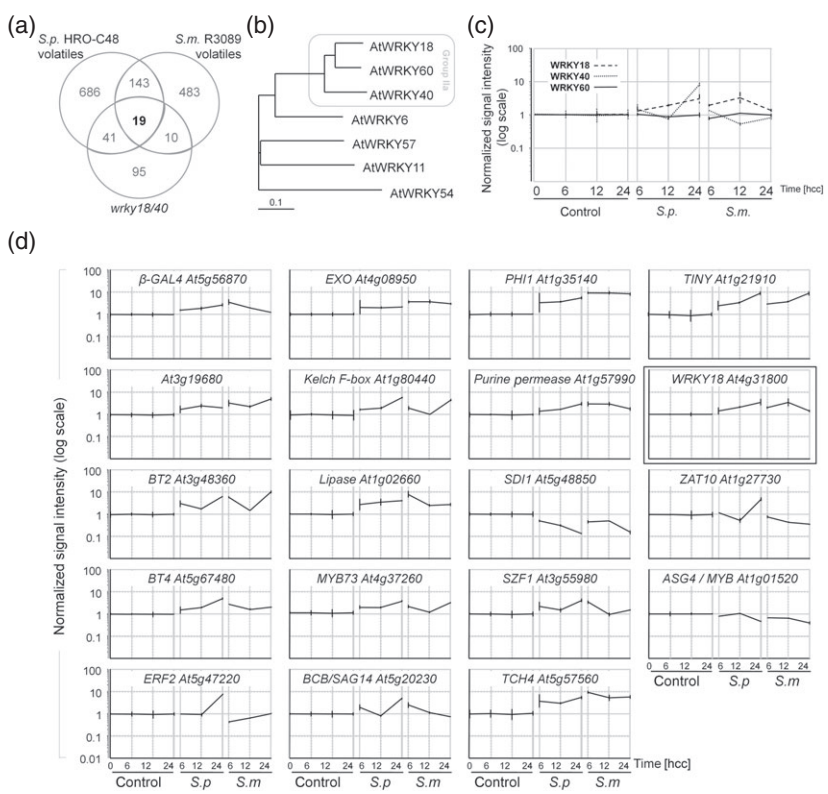

Figure 6. Comparison of volatile-induced gene expression with genes that are up- or down-regulated in the *wrky18/40* double mutant.
(a) Venn diagram of volatile-responsive genes and genes that are up- or down-regulated in the *wrky18/40* double mutant (Pandey *et al.*, 2010).
(b) Phylogenetic tree of paralogous group IIa WRKY protein sequences and more distantly related proteins.
(c) Expression profile of *WRKY18, WRKY40* and *WRKY60* upon co-cultivation with *Se. plymuthica* or *St. maltophilia* in the microarray experiment.
(d) Expression profiles of the 19 genes (Table 3) that were differentially expressed in the microarray experiments under all three conditions (see Figure 6a).

redundant with its paralogs AtWRKY40 and AtWRKY60 (Figure 6b) (Xu *et al.*, 2006).

Of the 165 genes up- or down-regulated in the *wrky18/40* double mutant compared to wild-type (Pandey *et al.*, 2010), 70 were also volatile-responsive (Figure 6a): 41 genes were shared for the *Se. plymuthica*-specific response ($P \leq 1.4 \times 10^{-39}$) and ten for the *St. maltophilia*-specific response ($P \leq 3.0 \times 10^{-11}$) (Figure 6a and Tables S4 and S5). Interestingly, ten of the 41 genes shared with the *Se. plymuthica* response were associated with ethylene

biosynthesis, signal transduction and signal integration (shown in red in Table S5).

Nineteen of the 162 commonly responding genes were also mis-expressed in the *wrky18/40* double mutant (Figure 6d). To determine whether these 19 genes are under the direct control of either WRKY18 or WRKY40, we examined the occurrence of W boxes in promoter sequences upstream of the genes and found a total of 54 W boxes (a mean of 2.8 per promoter) (Table 3).

We next tested *wrky18*, *wrky40* and *wrky60* single mutant lines in triple culture with wild-type plants. Only *wrky18* displayed significant differences compared to wild-type (Figure 7): its fresh weight was almost twofold higher (Figure 7b) and the mutants were much less chlorotic after 7 days of co-cultivation (Figure 7a,c). The relative fresh weight of *wrky18* mutants increased to more than three times their starting weight after 10 days of co-cultivation (Figure S4). However, *wrky18* plants could not be rescued by transferring to MS plates after 10 days of co-cultivation and chlorophyll was hardly detectable (Figure 7a). The *wrky40* and *wrky60* mutants did not exhibit significant phenotypic differences compared to wild-type in co-cultivation with either bacteria (Figure 7b,c and Figure S5). As *wrky18* mutants were less affected by the bacterial volatiles, we investigated whether its H_2O_2 accumulation was impaired. That, however, was not the case (Figure 7d and Figure S5).

DISCUSSION

Plants' responses to inhibiting volatiles emitted by *Se. plymuthica* HRO-C48 and *St. maltophilia* R3089 were studied by transcriptomic analysis and verified by quantitative RT-PCR for selected marker genes (Tables S6 and S7). The impact of microbial volatiles on plant physiology has been underestimated for a long time (Wenke *et al.*, 2010), but it appears that volatiles may play a role in plant–pathogen interactions. In dual-culture, volatiles from both strains significantly decreased plant growth within 2–3 days, resulting in chlorosis and cell death (Wenke *et al.*, 2012). Removal of *Se. plymuthica* within 36 h of dual-culture, before H_2O_2 accumulated, resulted in a clear decrease in volatile-induced symptoms and plants were able to recover (Wenke *et al.*, 2012). At present, it is unclear whether reactive oxygen species (ROS) are directly or indirectly responsible as a signal leading to chlorosis. Microarray analysis was performed at 6, 12 and 24 h of dual-culture before induction of irreversible damage. Compared to classical (a)biotic stress experiments, these time points are considered late. However, plants are not immediately exposed to volatiles after application of bacteria, as the volatiles have to disperse within Petri dishes. Furthermore, the volatiles do not activate the *GST1* box before 18 h of dual-culture, whereas this element is rapidly responsive to pathogens (Rushton *et al.*, 2002). Transcription and translation of factors downstream of an immediate early signaling cascade are considered to be responsible for activation of this reporter gene. Therefore, we assumed that such signaling must occur prior to *GST1* dependent reporter activation, and thus timed our experiments accordingly.

The experimental system used is a simple standardized system to determine the effect of volatiles on plants.

Table 3 Genes that were up- or down-regulated in the *wrky18/40* double mutant and responded to both *Se. plymuthica* and *St. maltophilia* volatiles

AGI	Description	Number of W boxes
At1g21910	AP2 domain-containing transcription factor family protein	3
At5g56870	β-galactosidase, putative/lactase, putative	5
At5g47220	Ethylene-responsive element-binding factor 2 (ERF2)	4
At3g19680	Expressed protein	2
At1g80440	Kelch repeat-containing F-box family protein	1
At1g02660	Lipase class 3 family protein	0
At5g48850	Male sterility MS5 family protein	9
At1g01520	Myb family transcription factor	0
At4g37260	Myb family transcription factor (MYB73)	3
At1g35140	Phosphate-responsive protein, putative	2
At4g08950	Phosphate-responsive protein, putative (EXO)	2
At5g20230	Plastocyanin-like domain-containing protein	2
At1g57990	Purine permease-related	3
At3g48360	Speckle-type POZ protein-related	0
At5g67480	TAZ zinc finger family protein/BTB/POZ domain-containing protein	2
At4g31800	WRKY family transcription factor	4
At5g57560	Xyloglucan endotransglucosylase TOUCH 4 (TCH4)	3
At1g27730	Zinc finger (C_2H_2 type) family protein (ZAT10)	4
At3g55980	Zinc finger (CCCH-type) family protein	5

The number of W boxes is given for the −1500 bp sequence upstream of the translation start site (ATG).

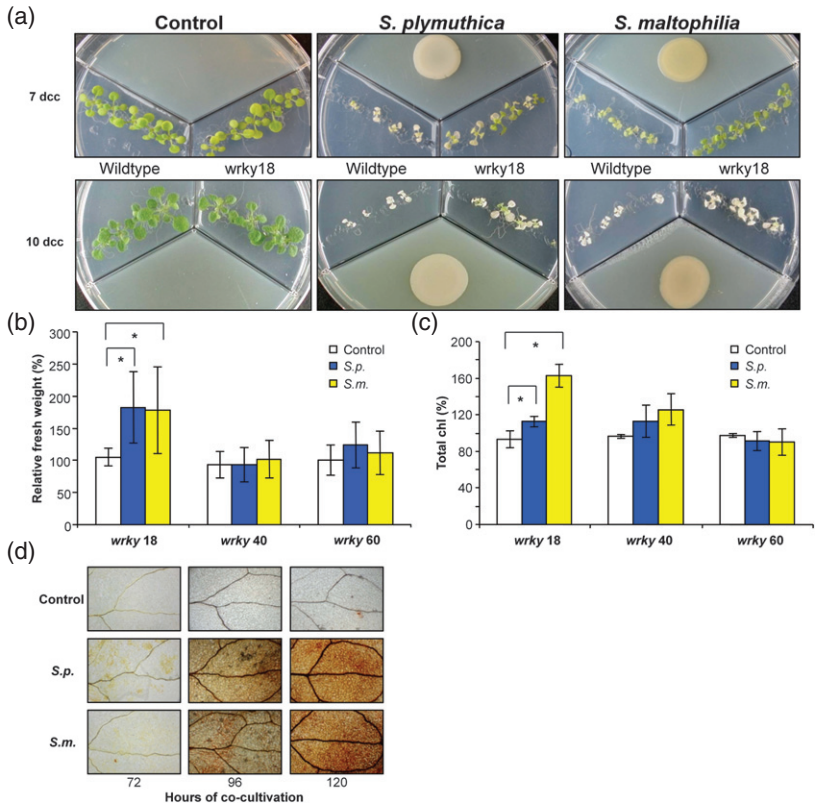

Figure 7. Co-cultivation of *WRKY* mutants.
(a) Wild-type and *wrky*18 mutant plants co-cultivated with *Serratia plymuthica* and *Stenotrophomonas maltophilia* compared to control after 7 and 10 days of co-cultivation.
(b) Relative fresh weight of *wrky* mutants relative to wild-type plants in the same triple culture after 7 days of co-cultivation (n = 9–12).
(c) Relative chlorophyll *a* and *b* content in *wrky* mutants relative to wild-type plants in the same triple culture after 7 days of co-cultivation (n = 3).
(d) Diaminobenzidine staining of cotyledons of *wrky18* mutants co-cultivated with *Se. plymuthica* or *St. maltophilia* compared to control. H_2O_2 reacts with diaminobenzidine to form a brownish precipitate.
Asterisks in (b) indicate $P \leq 0.01$; asterisks in (c) indicate $P \leq 0.05$ (paired Student's t test).

Importantly, dual-cultures (unsealed) allow gas exchange with the ambient air (Kai and Piechulla, 2009). In dual-culture, bacteria and plants communicate only via volatiles, in contrast to direct physical contacts. In other studies, *Se. plymuthica* HRO-C48 exhibits antagonistic properties against soil-borne plant pathogens and plant growth-promoting effects (Kalbe *et al.*, 1996; Berg, 2000), and *St. maltophilia* R3089 produces the anti-fungal agent maltophilin (Jakobi and Winkelmann, 1996). To fully understand the complex interactions between bacteria and plants, all aspects of this interplay need to be studied.

The nature of bioactive volatiles is still elusive (Stall *et al.*, 1972; Lovrekovich *et al.*, 1969; Etschmann *et al.*, 2002). For microarray analysis, we decided to use volatiles emitted by living rhizobacteria rather than synthetic compounds for three reasons: (i) it is more similar to nature, (ii) only few bio-effective volatiles have been identified and are presently available, and (iii) synergistic or additive effects are expected to occur as it has been speculated for dimethyldisulfide and ammonia emitted by *Serratia odorifera* 4Rx13 (Kai *et al.*, 2010).

The only volatile commonly emitted by *Se. plymuthica* and *St. maltophilia* identified to date is 2-phenylethanol (Kai *et al.*, 2007; Kai and Piechulla, 2008). It has been described as affecting biological membranes (transport systems for sugars and amino acids, permeability) (Ingram and Buttke, 1984; Seward *et al.*, 1996; Etschmann *et al.*, 2002). Application of 2-phenylethanol led to significant growth inhibition of Arabidopsis, with 50% inhibition at a concentration of 20 μmol (Figure S6). Whether such amounts are emitted in dual-culture with *Se. plymuthica* and *St. maltophilia* cannot be answered at present. Sodorifen (Von Reuss *et al.*, 2010) was shown to be the major component emitted by *Serratia* spp. (Kai *et al.*, 2007), but had no effect on Arabidopsis (Kai *et al.*, 2010). Other than 2-phenylethanol, the volatile profiles of *Se. plymuthica* and *St. maltophilia* are quantitatively/qualitatively different. Thus, the strain specificity of the kinetics of growth inhibition and transcriptional changes comes as no surprise. The different cell numbers at 6 and

12 h of dual-culture (Figure S1) contribute to a presumably neglible extent. Volatile emission is partly dependent on bacterial growth stage (Bunge *et al.*, 2008; Kai *et al.*, 2010), but both bacteria simultaneously enter the stationary phase after 24 h of dual-culture with almost the same number of colony-forming units.

Most of the *St. maltophilia* volatile-responsive genes were regulated at 6 h of dual-culture but the main transcriptional response to *Se. plymuthica* was detected at 24 h of dual-culture. These results conflict to an extent with morphological and physiological observations. Repression of genes encoding components of the mitochondrial electron transport chain and photosystems by *St. maltophilia* volatiles may partially explain the delayed ROS activation and may be the characteristics of a specific response. At 18 h of dual-culture the *GST1* element was induced with comparable kinetics by both rhizobacteria. Interestingly, the *GST1* element contains a W box and an S box, which are essential for pathogen responsiveness and during senescence (Rushton *et al.*, 2002), such that the 4x*GST1* GUS reporter construct is under the control of WRKY and presumably AP2/ERF TFs (Rushton *et al.*, 1996, 2002). Therefore, we conclude that activation of putative downstream target genes by WRKY or AP2/ERF TFs also occurs within 18 h of dual-culture, or less (Figure 2b). Activation of *GST1* within 18 h of dual-culture indicates that sensing of the volatile signal was probably mediated via either the W box or the S box more than 1 or 2 days prior to ROS accumulation in response to *Se. plymuthica* and *St. maltophilia* volatiles, respectively. ROS are one of the earliest signals produced in response to pathogens (Lamp and Dixon, 1997). The late and long-lasting volatile-induced ROS accumulation highlights clear differences in biotically induced H_2O_2 production, as confirmed by the limited overlap between volatile- and oxidative stress-regulated genes. As H_2O_2 also accumulates during various abiotic stresses (Neill *et al.*, 2002; Mittler *et al.*, 2004), ROS production is considered to be a non-specific alarm signal. Therefore, it cannot be determined which route of reaction (biotic or abiotic) is activated by bacterial volatiles. However, microarray analysis indicates the involvement of several hormones in ROS production: auxin (Schopfer, 2001), ethylene (Mehlhorn, 1990), gibberellic acid and ABA (reviewed by Kwak *et al.*, 2006). Cross-talk between auxin and ethylene has been proposed to lead to ABA-mediated H_2O_2 production (Kraft *et al.*, 2007; Grossmann *et al.*, 2001).

Several results have suggested that volatile sensing and signal transduction involves WRKY18 function. First, the affected genes are enriched for W boxes, the *cis*-element target of WRKYs (Table 2). Second, the 4x*GST1* element responded to volatiles and contains W boxes (Figure 2b). Third, *WRKY18* was significantly regulated by both bacterial volatiles (Figure 6c and Table S6). Indeed, the *wrky18* mutant showed a more volatile-tolerant phenotype than wild-type, *wrky40* or *wrky60* at 7 and 10 days of co-cultivation for both bacteria (Figure S4). WRKY18 is known to be involved in pathogen responses (Pandey *et al.*, 2010), but transgenic plants with high transcript levels of *WRKY18* showed stunted growth (Chen and Chen, 2002). WRKY18 acts redundantly with its paralogs WRKY40 and WRKY60 (Xu *et al.*, 2006). Due to their high similarity, all three WRKY factors display genetic, physiological and physical interactions, and possibly control expression of the same downstream target genes (Xu *et al.*, 2006; Wang *et al.*, 2006; Shen *et al.*, 2007; Chen *et al.*, 2010). More recently, transcriptional reprogramming by WRKY18 and WRKY40 after powdery mildew infection has been successfully investigated (Shen *et al.*, 2007; Pandey *et al.*, 2010). WRKY18 and WRKY40 are known to work antagonistically at times (Xu *et al.*, 2006; Chen *et al.*, 2010). In dual-culture, *wrky18* mutants exhibited delayed growth inhibition and chlorosis; however, all *wrky18*, *wrky40* and *wrky60* mutant plants ultimately died, suggesting that WRKY18-dependent resistance is only partially responsible for the phenotype.

Previous studies on WRKY18 focused on a role in defense. WRKY18 was found to play a role in activation of systemic acquired resistance, with NPR1 as its direct target (Wang *et al.*, 2006). This interaction led to greater resistance to *Pseudomonas syringae* in lines over-expressing *wrky18* (Xu *et al.*, 2006). In contrast, WRKY18/40 function was independent of the NPR1/salicylic acid (SA) pathway during powdery mildew infection (Pandey *et al.*, 2010). Similarly, we did not detect any changes in NPR1 or other SA marker gene expressions (Figure S7), as confirmed by the limited overlap in meta-analysis with patterns obtained after infection with avirulent (avrRpm1) and virulent (DC3000) strains of *P. syringae* (Grant *et al.*, 2006) (Figure 7) and by the lack of regulation (*St. maltophilia*) or down-regulation (*Se. plymuthica*) of SA signaling (Figure 5).

Our meta-analysis revealed strong overlap with ABA and methyl jasmonate treatments, both of which have been shown to involve WRKY18 (Chen *et al.*, 2010; Shang *et al.*, 2010; Wang *et al.*, 2008; Pandey *et al.*, 2010), but no typical ABA or methyl jasmonate marker genes were regulated in response to the volatiles (Figure S7). To identify possible target genes of WRKY18, we compared genes affected in the *WRKY18/40* double mutant relative to wild-type (Pandey *et al.*, 2010) and the 162 commonly regulated genes. We found 19 candidates, nine of which are TFs, that were generally enriched in W boxes (Table 3). WRKY40 has been shown to bind to the promoters of the AP2-type TF gene *RRTF1* and *JAZ8*, a JA signaling repressor (Pandey *et al.*, 2010), whereas WRKY18 was identified as positive regulator of JA (Wang *et al.*, 2008). We did not observe a response of *RRTF1* or *JAZ8* due to volatiles (Figure S8). However, the 21 TFs in the 162 commonly regulated genes included three AP2 domain-containing proteins, six MYB domain-containing proteins and WRKY18 (Table 1). These three protein

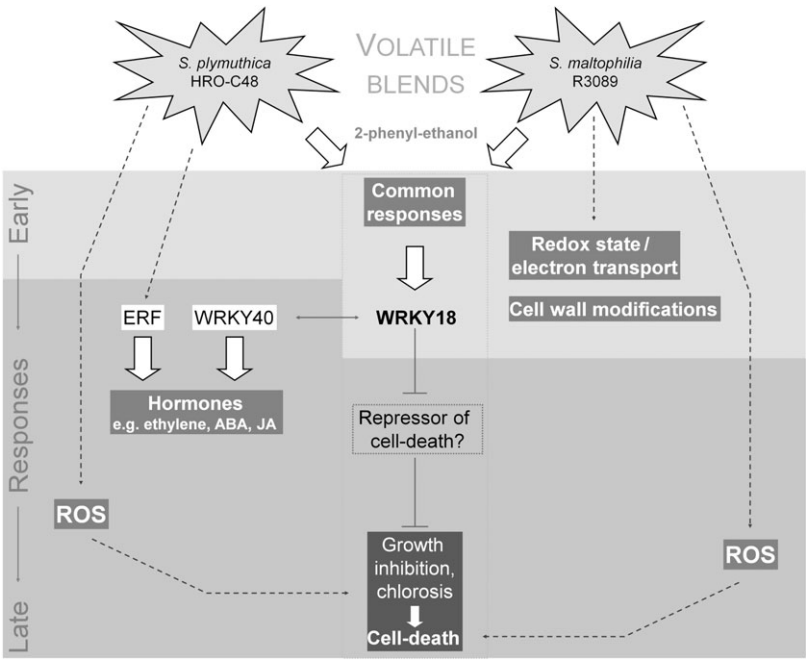

Figure 8. Schematic overview of volatile-triggered responses.
Serratia plymuthica- or *Stenotrophomonas maltophilia*-specific and common pathways are shown at early or late phases of response. WRKY18 is a central mediator of volatile-induced cell death. Its known function as transcriptional repressor positions it upstream of a putative repressor of cell death. *Se. plymuthica*-specific responses involved ethylene response factors (ERFs) and WRKY40, a paralog of WRKY18. Involvement of hormones, redox state / electron transport and cell-wall modifications is deduced from functional categories and cellular processes. ROS, reactive oxygen species.

families have been shown to contain interacting members that work together in diverse signaling pathways during common stress responses (Ma and Bohnert, 2007). It would be interesting to test whether these genes are directly involved in volatile responses.

Finally, we have summarized our findings to provide an overview of the time frame of responses to *Se. plymuthica* and *St. maltophilia* volatiles (Figure 8). We found that volatile-triggered responses common to both rhizobacteria and strain-specific processes show different kinetics. Microarray analysis provided evidence of the involvement of ethylene, ABA and JA, as well as upstream regulators such as ERFs and WRKY40, at later time points of *Se. plymuthica* co-cultivation. In contrast, relatively rapid changes in redox potential and electron transport occurred during the *St. maltophilia* response. Although cell-wall modifications specifically accompanied the early *St. maltophila* response, expression changes of individual genes, such as the stress responsive xyloglucan endotransglucosylase TOUCH 4 (TCH4) gene, were common to both strains. Different responses can be explained by the mixtures of volatiles released, while responses common to both strains were possibly induced by identical or highly similar classes of volatile metabolites (Wenke *et al.*, 2012).

Given that WRKY18 functions as a repressor of gene expression and acts antagonistically on *WRKY40* expression (Xu *et al.*, 2006; Shen *et al.*, 2007; Chen *et al.*, 2010; Pandey *et al.*, 2010), the significantly reduced growth inhibition of the *wrky18* mutant allowed us to propose a double-negative signaling cascade. This type of cascade would require an as yet unknown factor that putatively represses cell death. Candidates for such a repressor function could be the sulphate deficiency-induced SULPHATE DEFICIENCY-INDUCED 1 (*SID1*, At5g48850) or MYB (At1g01520) genes, which were up- or down-regulated in the *wrky18/40* double mutant (Pandey *et al.*, 2010) and repressed by volatiles from both species (Figure 6d). In the *wrky18* mutant, suppression of this factor is lifted, resulting in de-repression of cell death. Apparently, this derepression prolonged the plant's vital phase. Interestingly, ROS accumulated in both mutants and wild-type (Figure S5), implying that rhizobacteria-specific processes also induced ROS in a separate signaling cascade that finally triggered non-specific cell death in Arabidopsis in a WRKY18-independent manner.

This study focused on a new type of plant stress elicitors that are derived from microbes and are transmissible through airspace. Previous studies on stress responses have investigated direct physical plant–pathogen contact or (a)biotically induced damage. Sensitively balanced and coordinated mechanisms induced by exo/endogenous elicitors, such as pathogen-associated molecular patterns (PAMPs), microbe-associated molecular pattern (MAMPs), effectors or more general damage-associated molecular pattern (DAMPs), result in local and/or systemic pathogen

resistance and wound healing (reviewed by Chisholm *et al.*, 2006; Lotze *et al.*, 2007; Boller and Felix, 2009). Importantly, meta-analysis uncovered much greater similarities between volatile responses and direct pathogen contact (avrRpm1, DC3000, HrcC, Psph, Pi) compared to PAMPs/MAMPs (Flg22, HrpZ, LPS, NPR1). This suggests the involvement of bacterial volatiles during pathogen attack. Consequently, aspects of pathogen-responsive signaling (e.g. the WRKY18-dependent pathway) may be due to volatile action. To differentiate, microbial volatiles from known PAMPs/MAMPs, we propose the term 'microbial volatile-associated molecular pattern' (mVAMP) for this type of volatile elicitor emitted by microbes. Based on ongoing experiments, we propose that bacterial volatiles have multiple effects in dual-culture and under natural conditions (e.g. Kai *et al.*, 2008). The use of volatiles from two rhizobacteria confirmed the strain specificity of early reactions, and underlined the importance of microbial volatiles as elicitors of responses to pathogens.

EXPERIMENTAL PROCEDURES

Co-cultivation of Arabidopsis with rhizobacteria

Arabidopsis thaliana seeds were surface-sterilized (2 min in 70% ethanol, 5 min in 1% calcium hypochlorite), rinsed four times in sterile dH_2O, and placed on half-strength Murashige and Skoog salt (MS) solid medium. Experiments with wild-type (Col-0) and 4x*GST1* GUS reporter lines (Rushton *et al.*, 2002) were performed in dual culture with plants being grown on MS medium and bacteria being grown on nutrient broth solid medium (Figure 1a). *wrky* mutants (Xu *et al.*, 2006) were analyzed in triple cultures with wild-type plants. After stratification (3 days at 4°C), plants were grown at 22 ± 2°C and 100 ± 10 μE m^{-2} sec^{-1} (16 h light and 8 h dark) at a relative humidity of 50 ± 10%. Three-day-old seedlings at equal developmental stages were selected, and the rest of the seedlings were removed from the split-dish plates. On the same day, the nutrient broth medium was inoculated with *St. maltophilia* R3089 or *Se. plymuthica* HRO-C48, which were originally isolated from the rhizosphere of *Brassica napus* L. ssp. *oleifa* (Berg and Ballin, 1994; Kalbe *et al.*, 1996).

At 4 days after germination, co-cultivation was started by adding 10^7 colony-forming units of rhizobacteria in liquid NBII medium or the same amount of liquid NBII medium (control) to solid NBII medium for dual-culture. The number of colony-forming units was determined by measuring the optical density at 600 nm before starting co-cultivation and by performing serial dilutions.

Plant phenotype and statistics

Seedlings from one plate were pooled to create one biological replicate. Biological replicates were performed in independent experiments. Plant growth of the wild-type, mutant and transgenic reporter lines was quantified on the basis of the length of cotyledons and primary root within 5 days of co-cultivation. At the 7th and 10th days of co-cultivation, the fresh weight of mutants and wild-type from triple cultures was determined. Additionally, photographs of seedlings were taken using an Olympus C-3030 digital camera (http://www.olympus-global.com/). Chlorophyll content was quantified as described by Porra *et al.* (1989). Relative values and paired *t* test-based significances were calculated using Microsoft Excel (http://www.microsoft.com/). Conditions for the *t* tests were checked by ANOVA using MeV software (Saeed *et al.* 2003, 2006).

Detection of hydrogen peroxide

H_2O_2 was detected using 3,3′-diaminobenzidine (Thordal-Christensen *et al.*, 1997) in three independent experiments. Cotyledons were cut and vacuum-infiltrated in diaminobenzidine buffer. After 2 h, chlorophyll was extracted in boiling ethanol (96%) for 10 min. Samples were stored in 96% ethanol and examined by light microscopy (Olympus BX41).

Promoter GUS assay

In two independent experiments, each performed in duplicate, seedlings were harvested and used for histochemical GUS staining by X-Gluc (Jefferson *et al.*, 1987). Additionally, cotyledons of three dual-cultures were pooled to create one biological replicate, and triplicate samples were prepared for fluorometric assays (Jefferson *et al.*, 1987).

Microarray hybridization

For each of two biological replicates, seedlings of five Petri dishes were treated, harvested and immediately frozen in liquid nitrogen. RNA was extracted using a Qiagen RNeasy plant mini kit (http://www.qiagen.com/) according to the manufacturer's instructions. Precipitation of nucleic acids was performed using 8 μl 3 M sodium acetate (pH 5.2), 2 μl glycogen (5 mg/ml) and ethanol (100%). After storing at −80°C and centrifugation (30 min, 13 000 *g*, 4°C), the pellet was rinsed twice with 500 μl ethanol (70%). The purified RNA was dissolved in diethyl pyrocarbonate-treated water. RNA quantity and integrity were assessed using an RNA 6000 Nano LabChip kit and BioAnalyzer software (Agilent Technologies, http://www.agilent.com).

Independent duplicates of 1 μg total RNA were used for cRNA synthesis (MessageAmp™ II-Biotin Enhanced single-round aRNA amplification kit, Ambion http://www.invitrogen.com/site/us/en/home/brands/ambion.html). The cRNAs were fragmented using RNA fragmentation reagent (Ambion), and hybridized to an Arabidopsis ATH1 chip (Affymetrix, http://www.affymetrix.com/). Hybridization, staining and scanning were performed according to the manufacturer's instructions.

Data processing and statistics

The Affymetrix CEL files were imported into GeneSpring software version 7 (Agilent Technologies). Per-chip normalization to the median was applied to achieve comparability. Arrays were adjusted for background optical noise using robust multiarray averaging (RMA) software, and normalized using quantile normalization (Wu *et al.*, 2004). Genes for which signal intensities in both treatment replicates were at least twofold higher or lower than signal intensities for both control replicates were classified as up- or down-regulated, respectively.

The data discussed here have been deposited in the National Centre for Biotechnology Information Gene Expression Omnibus (Edgar *et al.*, 2002), and are accessible through Gene Expression Omnibus series accession number GSE35325 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35325).

Functional categorization using gene ontology (GO) terms was performed using http://www.arabidopsis.org/tools/bulk/go/ jsp (Swarbreck *et al.*, 2008) with *Arabidopsis thaliana* genome release 9. Percentages of all functional categories in the various gene lists were calculated using Excel. For significant over-representation or under-representation of a particular GO category, *P* values were calculated using hypergeometric distribution in Microsoft Excel

(http://www.microsoft.com). GO categories of the universe (all genes present on the ATH1 array) were compared with the sampling distribution of respective gene lists.

MapMan software version 3.0.0 (http://gabi.rzpd.de/projects/MapMan/) was used with default settings to visualize functional BINs of volatile-responsive genes. Background-corrected RMA data for the 1382 differentially expressed probe sets were normalized to controls under the following conditions: *Se. plymuthica* at 6, 12 and 24 h, or *St. maltophilia* at 6, 12 and 24 h. Adjusted values were imported into the R software package (http://cran.r-project.org/) to produce MapMan-compatible inputs. Default settings were used to import data.

Meta-data analysis and statistics

For comparison of expression information with other microarray experiments provided by AtGenExpress (Wanke *et al.*, 2009), Affymetrix CEL files ME00325 (cold), ME00326 (genotoxic stress), ME00327 (osmotic stress), ME00328 (salt stress), ME00329 (UV-B stress), ME00330 (wounding), ME00338 (drought), ME00339 (heat), ME00340 (oxidative stress), ME00332 (response to bacterial and oomycete-derived elicitors), ME00331 (response to virulent, avirulent, type III secretion system-deficient and non-host bacteria), ME00333 (response to abscisic acid), ME00334 (response to 1-aminocyclopropane-1-carboxylic acid), ME00335 (response to brassinolides), ME00336 (response to indole-3-acetic acid), ME00337 (response to methyl jasmonate), ME00343 (response to gibberellic acid) were retrieved from the Arabidopsis Information Resource (http://www.arabidopsis.org) (Swarbreck *et al.*, 2008). Cell culture results were excluded from heat stress data (ME00339). Data were evaluated as described by Kilian *et al.* (2007). Definition of differentially expressed genes required at least a threefold difference from mean expression under at least one condition. Data for growth-promoting *Bacillus subtilis* GB03 volatile-responsive genes were taken from Zhang *et al.* (2007). Data for *wrky18/40* double mutants were taken from Pandey *et al.* (2010).

The resulting datasets were subsequently compared to the volatile-responsive genes identified here. Significant overlaps between datasets of various sizes were calculated using a hypergeometric distribution.

cis-element analysis

Analysis of elements in promoters of differentially regulated genes was performed using Athena (O'Connor *et al.*, 2005). Significant enrichment of motifs was calculated using hypergeometric distribution in 1500 bp upstream of the ATG codon. Promoter sequences were retrieved from the RSA (Regulatory Sequence Analysis, http://rsat.scmbb.ulb.ac.be/rsat/) tools webpage (Thomas-Chollier *et al.*, 2008).

Phylogenetic tree

The amino acid sequences of the depicted WRKY proteins (Figure 6b) were retrieved from the Arabidopsis Information Resource (http://www.arabidopsis.org), and were used to create a multiple protein sequence alignment-based phylogram using default settings in ClustalW (http://www.ebi.ac.uk/clustalw/) (Thompson *et al.*, 1994).

Quantitative RT-PCR

In an independent experiment, for each of two biological replicates, seedlings from five dual-culture were treated, harvested and immediately frozen in liquid nitrogen. RNA was extracted using a Qiagen RNeasy plant mini kit according to the manufacturer's instructions. RNA samples were reversed-transcribed into cDNA using SuperScript reverse transcriptase (Invitrogen, http://www.invitrogen.com/) and oligo(dT) primers. cDNA was quantified using gene-specific primers (Bio-Rad Laboratories, http://www.bio-rad.com/) (Table S8). PCR was performed on an iQ5 real-time PCR detection system (Bio-Rad Laboratories). *Actin7* (At5g09810) and *Ubiquitin10* (At4G05320) served as internal controls. Data were analysed by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

ACKNOWLEDGMENTS

We thank Markus Schmid for support with microarray hybridizations and Imre E. Somssich for providing seeds of *wrky* mutant lines. We acknowledge the Deutsche Forschungsgemeinschaft for funding the publicly available AtGenExpress experiments. Furthermore, we thank Diana Sonnenburg for performing chlorophyll quantification of wild-type plants. We are grateful for the many helpful suggestions of Marco Kai. This work was supported by the Deutsche Forschungsgemeinschaft (BP 153/26-1 and HA2146/10-1).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Number of colony-forming units in dual and triple cultures.
Figure S2. Expression trajectories of differentially expressed genes.
Figure S3. Metabolic overview of volatile-responsive genes.
Figure S4. Relative fresh weight of *wrky* mutants.
Figure S5. Diaminobenzidine staining of *wrky* mutants.
Figure S6. Effect of 2-phenylethanol.
Figure S7. Expression trajectories of SA response and ABA response marker genes.
Figure S8. Expression trajectories of WRKY18 target genes.
Table S1. Genes responsive to volatiles from both species.
Table S2. Genes specifically responsive *St. maltophilia* volatiles.
Table S3. Genes specifically responsive to *Se. plymuthica* volatiles.
Table S4. Genes specifically responsive to *St. maltophilia* volatiles and affected in the *wrky18/40* double mutant.
Table S5. Genes specifically responsive to *Se. plymuthica* volatiles and affected in the *wrky18/40* double mutant.
Table S6. Expression analysis of *wrky18*, *wrky40* and *wrky60* mutants by microarray analysis and quantitative RT-PCR.
Table S7. Expression analysis of selected marker genes by microarray analysis and quantitative RT-PCR.
Table S8. Primer sequences for quantitative RT-PCR.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

Alström, S and Burns, R.G. (1989) Cyanide production by rhizobacteria as a possible mechanism of plant growth inhibition. *Biol. Fertil. Soils*, **7**, 232–238.

Barber, DA and Martin, JK (1976) The release of organic substances by cereal roots into soil. *New Phytol.* **76**, 69–80.

Berardini, TZ, Mundodi, S, Reiser, R *et al.* (2004) Functional annotation of the Arabidopsis genome using controlled vocabularies. *Plant Physiol.* **135**, 1–11.

Berg, G (2000) Diversity of antifungal and plant-associated *Serratia plymuthica* strains. *J. Appl. Microbiol.* **88**, 952–960.

Berg, G and Ballin, G (1994) Bacterial antagonists to *Verticillium dahliae*. *J. Phytopathol.* **141**, 99–110.

Boller, T and Felix, G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* **60**, 379–406.

Bunge, M, Araghipour, N, Mikoviny, T, Dunkl, J, Schnitzhofer, R, Hansel, A, Schinner, F, Wisthaler, A, Margesin, R and Märk, TD (2008) On-line monitoring of microbial volatile metabolites by proton transfer reaction-mass spectrometry. *Appl. Environ. Microbiol.* **74**, 2179–2186.

Chen, C and Chen, Z (2002) Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor. *Plant Physiol.* **129**, 706–716.

Chen, H, Lai, Z, Shi, J, Xiao, Y, Chen, Z and Xu, X (2010) Roles of Arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress. *BMC Plant Biol.* **10**, 281.

Chisholm, ST, Coaker, G, Day, B and Staskawicz, BJ (2006) Host–microbe interactions: shaping the evolution of the plant immune response. *Cell* **124**, 803–814.

Dietz, KJ, Jacquot, JP and Harris, G (2010) Hubs and bottlenecks in plant molecular signaling networks. *New Phytol.* **188**, 919–938.

Dong, J, Chen, C and Chen, Z (2003) Expression profiles of the Arabidopsis *WRKY* gene superfamily during plant defense response. *Plant Mol. Biol.* **51**, 21–37.

Edgar, R, Domrachev, M and Lash, AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* **30**, 207–210.

Etschmann, MMW, Bluemke, W, Sell, D and Schrader, J (2002) Biotechnological production of 2-phenylethanol. *Appl. Microbiol. Biotechnol.* **59**, 1–8.

Euglem, T and Somssich, IE (2007) Networks of WRKY transcription factors in defense signaling. *Curr. Opin. Plant Biol.* **10**, 366–371.

Eulgem, T, Rushton, PJ, Robatzek, S and Somssich, IE (2000) The WRKY superfamily of plant transcription factors. *Trends Plant Sci.* **5**, 199–206.

Fukuda, H, Ogawa, T and Tanase, S (1993) Ethylene production by microorganisms. *Adv. Microb. Physiol.* **35**, 275–306.

Goda, H, Sasaki, E, Akiyama, K *et al.* (2008) The AtGenExpress hormone and chemical treatment data set: experimental design, data evaluation, model data analysis and data access. *Plant J* **55**, 526–542.

Grant, SR, Fisher, EJ, Chang, JH, Mole, BM and Dangl, JL (2006) Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. *Annu. Rev. Microbiol.* **60**, 425–449.

Grossmann, K, Kwiatkowski, J and Tresch, S (2001) Auxin herbicides induce H_2O_2 overproduction and tissue damage in cleavers (*Galium aparine* L.). *J Exp Bot* **52**, 1811–1816.

Hennig, L, Menges, M, Murray, JA and Gruissem, W (2003) Arabidopsis transcript profiling on Affymetrix GeneChip arrays. *Plant Mol. Biol.* **53**, 457–465.

Ingram, LO and Buttke, TM (1984) Effects of alcohols on microorganisms. *Adv. Microb. Physiol.* **25**, 253–300.

Jakobi, M and Winkelmann, G (1996) Maltophilin: a new antifungal compound produced by *Stenotrophomonas maltophilia* R3089. *J. Antibiotics.* **49**, 1101–1104.

Jefferson, RA, Kavanagh, TA and Bevan, MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.

Kai, M and Piechulla, B (2008) Dufte(nde) Rhizobakterien – Flüchtige Bekanntschaften im Untergrund. *BIOspektrum* **14**, 246–249.

Kai, M and Piechulla, B (2009) Plant growth promotion due to rhizobacterial volatiles – an effect of CO_2? *FEBS Lett.* **583**, 3474–3477.

Kai, M and Piechulla, B (2010) Impact of volatiles of the rhizobacteria *Serratia odorifera* on the moss *Physcomitrella patens*. *Plant Signal Behav.* **5**, 1–3.

Kai, M, Effmert, U, Berg, G and Piechulla, B (2007) Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Arch. Microbiol.* **187**, 351–360.

Kai, M, Vespermann, A and Piechulla, B (2008) The growth of fungi and *Arabidopsis thaliana* is influenced by bacterial volatiles. *Plant Signal Behav.* **3**, 1–3.

Kai, M, Haustein, M, Molina Martir, F, Petri, A, Scholz, B and Piechulla, B (2009) Bacterial volatiles and their action potential. *Appl. Microbiol. Biotechnol.* **81**, 1001–1012.

Kai, M, Crespo, E, Cristescu, SM, Harren, FJM and Piechulla, B (2010) *Serratia odorifera*: analysis of volatile emission and biological impact of volatile compounds on *Arabidopsis thaliana*. *Appl. Microbiol. Biotechnol.* **88**, 965–976.

Kalbe, C, Marten, P and Berg, G (1996) Members of the genus *Serratia* as beneficial rhizobacteria of oilseed rape. *Microbiol. Res.* **151**, 433–439.

Kilian, J, Whitehead, D, Horak, J, Wanke, D, Weinl, S, Batistic, O, D'Angelo, C, Bornberg-Bauer, E, Kudla, J and Harter, K (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J* **50**, 347–363.

Kraft, M, Kuglitsch, R, Kwiatkowski, J, Frank, M and Grossmann, K (2007) Indole-3-acetic acid and auxin herbicides up-regulate 9-*cis*-epoxycarotenoid dioxygenase gene expression and abscisic acid accumulation in cleavers (*Galium aparine*): interaction with ethylene. *J Exp Bot* **58**, 1497–1503.

Kwak, JM, Nguyen, V and Schroeder, JI (2006) The role of reactive oxygen species in hormonal responses. *Plant Physiol.* **141**, 323–329.

Lamp, C and Dixon, RA (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 251–275.

Livak, KJ and Schmittgen, TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ method. *Methods* **25**, 402–408.

Lotze, MT, Zeh, HJ, Rubartelli, A, Sparvero, LJ, Amoscato, AA, Washburn, NR, Devera, ME, Liang, X, Tör, M and Billiar, T (2007) The grateful dead: damage associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunol. Rev.* **220**, 60–81.

Lovrekovich, L, Lovrekovich, H and Goodman, RN (1969) The role of ammonia in wildfire disease of tobacco caused by *Pseudomonas tabaci*. *Phytopathology* **59**, 1713–1716.

Ma, S and Bohnert, HJ (2007) Integration of *Arabidopsis thaliana* stress-related transcript profiles, promoter structures, and cell-specific expression. *Genome Biol.* **8**, R49.

Mehlhorn, H (1990) Ethylene-promoted ascorbate peroxidase activity protects plants against hydrogen peroxide, ozone and paraquat. *Plant Cell Environ.* **13**, 971–976.

Mittler, R, Vanderauwera, S, Gollery, M and Van Breusegem, F (2004) Reactive oxygen gene network of plants. *Trends Plant Sci.* **9**, 490–498.

Neill, SJ, Desikan, R, Clarke, A, Hurst, RD and Hancock, JT (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J Exp Bot* **53**, 1237–1242.

O'Connor, TR, Dyreson, C and Wyrick, JJ (2005) Athena: a resource for rapid visualization and systematic analysis of Arabidopsis promoter sequences. *Bioinformatics* **21**, 4411–4413.

Pandey, SP, Roccaro, M, Schön, M, Logemann, E and Somssich, IE (2010) Transcriptional reprogramming regulated by WRKY18 and WRKY40 facilitates powdery mildew infection of Arabidopsis. *Plant J.* **64**, 912–923.

Porra, RJ, Thompson, WA and Kriedemann, PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta* **975**, 384–394.

Robert, HS, Quint, A, Brand, D, Vivian-Smith, A and Offringa, R (2009) BTB and TAZ DOMAIN scaffold proteins perform a crucial function in Arabidopsis development. *Plant J.* **58**, 109–121.

Rossel, JB, Wilson, PB, Hussain, D, Woo, NS, Gordon, MJ, Mewett, OP, Howell, KA, Whelan, J, Kazan, K and Pogson, BJ (2007) Systemic and intracellular response to photooxidative stress in Arabidopsis. *Plant Cell* **19**, 4091–4110.

Rushton, PJ, Torres, JT, Parniske, M, Wernert, P, Hahlbrock, K and Somssich, IE (1996) Interaction of elicitor-induced DNA binding proteins with elicitor response elements in the promoters of parsley *PR1* genes. *EMBO J.* **15**, 5690–5700.

Rushton, PJ, Reinstadler, A, Lipka, V, Lippok, B and Somssich, IE (2002) Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. *Plant Cell* **14**, 749–762.

Ryu, CM, Farag, MA, Hu, CH, Reddy, MS, Wie, HX and Pare, PW (2003) Bacterial volatiles promote growth in *Arabidopsis thaliana*. *Plant Physiol.* **134**, 1017–1026.

Saeed, AI, Bhagabati, NK, Braisted, JC, Liang, W, Sharov, V, Howe, EA, Li, J, Thiagarajan, M, White, JA and Quackenbush, J (2006) TM4 microarray software suite. *Meth. Enzymol.* **411**, 134–193.

Saeed, AI, Sharov, V, White, J *et al.* (2003) TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **34**, 374–378.

Sakamoto, H, Araki, T, Meshi, T and Iwabuchi, M (2000) Expression of a subset of the *Arabidopsis* Cys_2/His_2-type zinc-finger protein gene family under water stress. *Gene* **248**, 23–32.

Schopfer, P (2001) Hydroxyl radical-induced cell-wall loosening *in vitro* and *in vivo*: implications for the control of elongation growth. *Plant J.* **28**, 679–688.

Schulz, S and Dickschat, JS (2007) Bacterial volatiles: the smell of small organisms. *Nat. Prod. Rep.* **24**, 814–842.

Seward, R, Willetts, JM, Dinsdale, MG and Lloyd, D (1996) The effects of ethanol, hexan-1-ol, and 2-phenylethanol on cider yeast growth, viability, and energy status; synergistic inhibition. *J. Inst. Brew.* **102**, 439–443.

Shang, Y, Yan, L, Liu, ZQ *et al.* (2010) The Mg-chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. *Plant Cell* **22**, 1909–1935.

Shen, QH, Saijo, Y, Mauch, S, Biskup, C, Bieri, S, Keller, B, Seki, H, Ulker, B, Somssich, IE and Schulze-Lefert, P (2007) Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science* **315**, 1098–1103.

Stall, RE, Hall, CB and Cook, AA (1972) Relationship of ammonia to necrosis of pepper leaf tissue during colonization by *Xanthomonas vesicatoria*. *Phytopathology* **62**, 882–886.

Swarbreck, D, Wilks, C, Lamesch, P *et al.* (2008) The Arabidopsis Information Resource (TAIR): gene structure and function annotation. *Nucleic Acids Res.* **36**, D1009–D1014.

Thimm, O, Bläsing, O, Gibon, Y, Nagel, A, Meyer, S, Krüger, P, Selbig, J, Müller, LA, Rhee, SY and Stitt, M (2004) MapMan: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant J.* **37**, 914–939.

Thomas-Chollier, M, Sand, O, Turatsinze, JV, Janky, R, Defrance, M, Vervisch, E, Brohée, S and Helden, J (2008) RSAT: regulatory sequence analysis tools. *Nucleic Acids Res.* **36**, W119–W127.

Thompson, JD, Higgins, DG and Gibson, TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680.

Thordal-Christensen, H, Zhang, Z, Wei, Y and Collinge, DB (1997) Subcellular localization of H_2O_2 in plants, H_2O_2 accumulation in papillae and hypersensitive response during barley–powdery mildew interaction. *Plant J.* **11**, 1187–1194.

Vespermann, A, Kai, M and Piechulla, B (2007) Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. *Appl. Environ. Microbiol.* **73**, 5639–5641.

Von Reuss, S, Kai, M, Piechulla, B and Francke, W (2010) Octamethylbicyclo(3.2.1)octadienes from the rhizobacterium *Serratia odorifera*. *Angew. Chem. Int. Ed. Engl.* **122**, 2053–2054.

Wang, D, Amornsiripanitch, N and Dong, X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog* **2**, 1042–1050.

Wang, Z, Cao, G, Wang, X, Miao, J, Liu, X, Chen, Z, Qu, LJ and Gu, H (2008) Identification and characterization of COI1-dependent transcription factor genes involved in JA-mediated response to wounding in Arabidopsis plants. *Plant Cell Rep.* **27**, 125–135.

Wanke, D, Berendzen, K, Kilian, J and Harter, K (2009) Insights into the Arabidopsis abiotic stress response from the AtGenExpress expression profile dataset. In *Plant Stress Biology* (Hirt, H., ed.). Weinheim, Germany: Wiley-VCH Verlag, pp. 199–225.

Weingart, H and Völksch, B (1997) Ethylene production by *Pseudomonas syringae* pathovars *in vitro* and *in planta*. *Appl. Environ. Microbiol.* **63**, 156–161.

Wenke, K, Kai, M and Piechulla, B (2010) Belowground volatiles facilitate interactions between plant roots and soil organisms. *Planta* **231**, 499–506.

Wenke, K, Weise, T, Kai, M, Warnke, R and Piechulla, B (2012) Volatiles mediating information between bacteria and plants. In *Bio-Communication of Plants* (Witzany, G and Baluska, F, eds). Heidelberg, Germany: Springer-Verlag, pp. 327–347, in press.

Wu, Z, Irizarry, RA, Gentleman, R, Martinez Murillo, F and Spencer, F (2004) A model-based background adjustment for oligonucleotide expression arrays. *J. Am. Statist. Assoc.* **99**, 909–917.

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.

Zhang, H, Kim, MS, Krishnamachari, V *et al.* (2007) Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in Arabidopsis. *Planta* **226**, 839–851.