# Chapter 16
# The Effects of Volatile Metabolites from Rhizobacteria on *Arabidopsis thaliana*

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## 16.1 Introduction

In the eukaryotic world the release and detection of volatile substances, often referred to as odorous compounds, is a well-known and effective way to send messages and to gain information. Just think of the wonderful, classic fragrances released by blossoms to attract bees from a distance. People are also strongly attracted—or repulsed—by many odors or scents of blossoms. The food and perfume industries take advantage of the human (olfactory) sense of smell, for example, the aroma of cheese or wine or the scent of a favorite deodorant. The great advantage of such substances, called volatiles by scientists, is the very long distance over which especially the sessile plants can attract or repulse their interaction partners. Volatiles are characterized by their lipophilicity, a low molecular weight of less than 300 Da, a high vapor pressure above 0.01 kPa (at 20 °C), and low boiling points. These properties cause the compounds to evaporate or vaporize. Most of the volatiles described up to now are aromatic compounds, derivatives of fatty acids, and terpenoids.

Microorganisms, in particular about 350 bacterial species studied to date, are important producers of volatile substances. Many commonly used and well-known aromas and odors such as those of cheese and wine (e.g., Urbach 1997; Schreier 1980) have their origin in the prokaryotic world, also the earthy smell in a forest after a rain shower caused by the geosmin released by actinomycetes (Gerber and Lechevalier 1965). The qualitative and quantitative distribution of individual compounds sometimes extremely complex mixtures of volatiles is mainly determined by the metabolic capabilities and capacities of the bacterial species involved and the availability of nutrients in line with respective growth conditions (Stotzky

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and Schenck 1976; Fiddaman and Rossall 1994). Anywhere from a dozen to nearly 100 compounds are emitted, among others, by *Chondromyces crocatus*, *Carnobacterium divergens* 9P, *Streptomyces* sp. GWS-BW-H5, and *Serratia odorifera* 4Rx13 (Schulz et al. 2004; Ercolini et al. 2009; Dickschat et al. 2005; Kai et al. 2010). At present, nearly 770 bacterial volatiles have been identified as belonging to 48 different classes of compounds (Wenke et al. 2012b).

The quantitatively dominant compounds are alkenes, ketones, and terpenoids with 120–190 compounds each, followed by acids, benzenoids, esters, and pyrazines (60–80 compounds), and 30–40 are representatives of the aldehydes, ether compounds, and lactones. Bacterial species investigated so far, which emits volatile mixtures, is far below the number of microorganisms present on earth. As a result, the analysis and identification of bacterial volatiles continues to be an interesting and multifaceted field of research. A number of techniques have been developed to collect and detect volatile substances (e.g., the open VOC (volatile organic compound) collection system, Kai et al. 2007; the closed-loop-stripping device, Boland et al. 1984; solid-phase microextraction (SPME), Arthur and Pawliszyn 1990; gas chromatography/mass spectrometry (GC/MS); proton transfer reaction/MS (PTR/MS), Mayr et al. 2003, etc.). Each technique has its merits but only reveals part of the volatile spectrum in the form of actual quality and quantity (summarized in Wenke et al. 2012b).

## 16.2 Volatiles as Infochemicals in Soil

It is interesting and important not only to identify novel volatiles from bacteria, but in fact there is always the question of the potential ecological and/or physiological function of the emitted compounds. For their producers, the release of volatile metabolites means a “loss” of essential carbon, in part as extremely energy-rich compounds. The buzzword “talking tree” (Baldwin et al. 2006) makes clear in an exemplary way the ecophysiological potential of volatile emission. Up to now, the focus of research in this area was and is what happens above the soil surface. This has made it possible to accumulate an extensive knowledge of the effects of airborne signals in the atmosphere (e.g., van Dam et al. 2010; Müller and Hilker 2000; Piechulla and Pott 2003; Zangerl and Berenbaum 2009). For the most part, findings that volatile substances are also produced, released, and detected below the soil surface have been neglected (summarized in Wenke et al. 2010). The zone surrounding the roots, i.e., the rhizosphere (Barber and Martin 1976), is an attractive environment for numerous types of organisms such as microbes, arthropods, nematodes, amebas, and ciliates. This is due to the energy-rich root exudates (Wenke et al. 2010).

The major producers of volatile metabolites are roots of plants. Typical plant volatiles in the soil are 1,8-cineol, γ-terpinene, β-myrcene, α-pinene, β-phellandrene, and β-caryophyllene, of which some are of major ecological significance (summarized in Kai et al. 2009b; Wenke et al. 2010). Rasman and

colleagues (2005) described the emission of β-caryophyllene by the roots of maize (*Zea mays*) induced by feeding damage, which consequently attracted the nematode *Heterorhabditis megidis* to directly ward off the Western corn rootworm *Diabrotica virgifera virgifera*. This example shows that release of volatile chemicals is dependent on the physiological state of the plants, as was demonstrated using carrots (*Daucus carota spp. sativa*) that were undamaged or damaged mechanically or by feeding (Weissteiner and Schütz 2006). In addition to plants, soil fungi and rhizobacteria emit all sorts of volatiles such as fungal alcohols octanol, ethanol, β-phenylethanol, 1-octen-3-ol, and octenal, as well as the bacterial metabolites trimethylamine, cyclohexanol, dimethyl disulfide, 2,3-butanediol, geosmin, ethylene, and 2,5-dimethyl pyrazine, but also simple, inorganic compounds such as carbon dioxide, ammonia, and hydrogen cyanide (summarized in Kai et al. 2009b, 2010; Blom et al. 2011; Bernier et al. 2011). Fungal volatiles play a significant role not only in intraspecies communication, such as attracting mating partners, but also in attraction or repulsion of other fungal or plant species. In the latter case, this led to the use of the term "burned area" referring to the zone surrounding the host plants of truffles, where growth of herbaceous plants is most likely suppressed by the fungal volatiles (Pacioni 1991). With regard to the significance of rhizobacterial volatiles, it is assumed that they play a role in inter- and intraspecies communication or as signals between cells, which serve the disposal of excess carbon compounds, and may even affect the growth of other organisms (plants, bacteria, fungi, nematodes, amebas, ciliates). These effects may be positive as well as negative (Kai et al. 2009a). Around 40 years ago, Stall and colleagues (1972) demonstrated that the ammonia produced by *Xanthomonas vesicatoria* is involved in necrosis of infected pepper. On the other hand, hydrogen cyanide enables pseudomonads to impair root growth of *Lactuca sativa* seedlings (Alström and Burns 1989). The most recent data have demonstrated an ammonia-induced change in antibiotic resistance in gram-negative and gram-positive bacteria (Bernier et al. 2011).

## 16.3 Dual Culture Tests to Study the Effects of Volatile Compounds

The so-called dual culture system established in recent years to study the effects of bacterial volatiles on other organisms was used in this study because of its simplicity (Wenke et al. 2012a). In a two-chamber Petri dish, the rhizobacteria are separated from their respective interaction partner by a plastic barrier, which only permits exchange of volatile metabolites. Nonspecific binding of gaseous substances is ensured by adding active charcoal, in order to observe significant effects on reduction of growth (Vespermann et al. 2007). The advantages of this system, i.e., dual cultures in partitioned Petri dishes, is the use of synthetic growth media, and reduction to only two interaction partners, serve to minimize any

variables. This ensures a good reproducibility and evaluation of test results, both indispensable for transcriptome analysis. The test system used represents one segment of the spectrum of natural conditions. In-depth studies of volatile-induced inhibition of plants are still in its infancy; hence, a simple system is required to understand the basic relationships.

Initially the test system of Ryu and colleagues (2003) was employed, who described the strong growth-promoting impact of rhizobacterial volatile metabolites on plants. The studies revealed an enlargement of the leaf surface in *Arabidopsis thaliana* in response to volatiles from the species *Bacillus*, *Serratia*, *Pseudomonas*, and *Escherichia*. The single active substance was identified as 2,3-butanediol. Further research has also demonstrated a variety of positive effects of 2,3-butanediol or of other complex mixtures of bacterial volatiles on plants (1) initiation or increase of plant resistance to biotic (*Erwinia carotovora*, *Pseudomonas syringae*) and abiotic stress (salinity, desiccation, or osmotic stress); (2) physiological, metabolic, and morphological changes (cell-wall expansion, more chloroplasts, increased photosynthetic capacity, accumulation of starch and iron, changes in volatile emission, increased production of essential oils, alteration in primary metabolism); and (3) involvement of plant hormones (auxin, abscisic acid, ethylene) (Ryu et al. 2004; Zhang et al. 2007, 2008a, b, 2009, 2010; Cho et al. 2008; Xie et al. 2009; Banchio et al. 2009; Rudrappa et al. 2010; Kwon et al. 2010; Ezquer et al. 2010).

When evaluating the plant growth-promoting effects mentioned, it should be taken into account that most of the data was collected in a closed system. Kai and Piechulla (2009) were able to identify a clear correlation between growth promotion and $CO_2$ enrichment during cocultivation of plants and bacteria in a closed system. In open dual cultures, there was a possibility of indirect promotion of plant fitness by volatiles emitted due to inhibition of plant pathogenic fungi such as *Rhizoctonia solani* (Kai et al. 2007). *Serratia plymuthica* HRO-C48 and *Stenotrophomonas maltophilia* R3089 turned out to be two of the most effective organisms in the dual culturing system. At the same time, volatile mixtures from *S. plymuthica* HRO-C48 and *S. maltophilia* R3089 showed significant effect on *A. thaliana* seedlings (Vespermann et al. 2007).

Once the dual culturing system was established for in-depth investigations, the bacteria and plants were each applied in two equidistant straight lines. A given number of seedlings and a defined bacterial cell count of $10^7$ were chosen at zero point in time. This corresponds to the bacterial concentration of *S. plymuthica* HRO-C48 and *Stenotrophomonas maltophilia* found on 1 g of fresh roots from strawberry or rapeseed plants, respectively, under field conditions (Kurze et al. 2001; Berg et al. 1996). At the beginning of the experiment, $10^7$ cells were applied accordingly, whereas when between $10^5$ and $10^7$ colony-forming units of *S. plymuthica* HRO-C48 were applied in preliminary experiments, there were no clear differences in cotyledon or root length. In general, rhizobacteria reached values of up to $10^8$ CFU/g fresh weight of strawberry, potato, and rapeseed roots (Berg et al. 2002). The formation of biofilms on root surfaces with a very high

localized bacterial density also has been described by number of researchers (Bloemberg et al. 2000; Walker et al. 2004).

## 16.4 Inhibitory Effects of Volatiles from *S. plymuthica* HRO-C48 and *S. maltophilia* R3089 on *A. thaliana*

The observation that *S. plymuthica* HRO-C48 and *S. maltophilia* R3089 inhibited *A. thaliana* seedling growth under dual culture conditions has raised many questions. First and foremost, it is interesting to find out which signaling pathways are involved and whether they are similar to plant responses to pathogens, whether these effects at physiological and molecular levels are dependent on the bacterial species, or whether the toxicity of mixtures of volatiles is nonspecific.

### *16.4.1 Morphological and Physiological Changes in* A. thaliana *Under Dual Culture Conditions*

In response to bacterial volatiles, the wild-type seedlings of *A. thaliana* visibly showed a marked reduction in early vegetative growth and a distinct paling of the leaves. Determination of chlorophyll and carotenoid contents showed an earlier drop in carotenoid content, in comparison to chlorophyll content, in response to both volatile mixtures. Measurement of cotyledon and root lengths confirmed a significant inhibition after 2–3 days of exposure to volatiles *S. plymuthica* HRO-C48 and *S. maltophilia* R3089, whereas in both cases the underground portion of the plant was affected earlier or to a greater degree (Wenke et al. 2012a). This could be caused by more rapid growth of the roots. However, the possibility of qualitative and quantitative differences in the distribution of volatile substances between plant medium and airspace—due to divergent polarities and volatilities of individual components—should also be taken into account. Another source of speculation would be the recognition of effective volatiles via the roots, which has yet to be clarified.

As to growth and leaf pigmentation, the response to *S. maltophilia* volatiles was delayed by about one day. There have been reports that volatile emission is quantitatively dependent on the bacterial growth phase (Bunge et al. 2008; Kai et al. 2010). Bacterial growth in dual cultures was checked, and in both cases the bacteria were growing exponentially after 6 and 12 h and had entered the stationary phase with $10^{11}$ cells after 24 h (Wenke et al. 2012a). A comparison of the exact number of cells in correlation with the kinetics of morphological effects revealed that the viable cell count of *S. maltophilia* was lower after 6 and 12 h than that of *S. plymuthica*. Experiments with $10^0$–$10^7$ *S. plymuthica* cells revealed that reduction of leaf and root length depends to a certain extent on the number of bacteria

used (Wenke et al. 2012b). The more cells applied initially, the earlier were significant effects detectable, whereas differences were minimal at around $10^5$ bacterial cells or more (unpublished data). Since all of the experiments for this report began with $10^7$–$10^8$ cells of *S. plymuthica* HRO-C48 or *S. maltophilia* R3089 (time zero), it can be assumed that slight differences in viable cell counts of both rhizobacterial species during the first few days had no significant effect on inhibition of *A. thaliana*. It is more likely that species-specific differences in the bacterial volatile mixtures (Kai et al. 2007) were responsible for kinetic differences in growth inhibition.

The complete loss of pigmentation by the *A. thaliana* seedlings within a few days led to the assumption that the rhizobacterial volatiles were lethal to the plants. Evans blue, a dye that penetrates dead cells (Kim et al. 2003), was used to recognize the time and location of cell death events in the cotyledons. A pale, randomly distributed blue staining of cotyledon cells was observed after 3 days in response to *S. maltophilia* R3069 and *S. plymuthica* HRO-C48 exposure. After 5 days, dye penetrated the cells to a very high degree (Wenke et al. 2012b). There were no species-specific differences in the effect caused by the bacterial volatiles, namely, the complete death of the seedlings in dual cultures after a continuous 5-day cocultivation of the plants with volatile infochemicals.

In an experiment in which the bacteria were removed from the coculture after different time periods, there was an enormous reduction in growth inhibition when the plants were only exposed to the volatile metabolites for up to 36 h (Wenke et al. 2012b). This implies that signaling pathways leading to a drastic inhibition of growth and to cell death had not been activated within this incubation period.

The type of stress caused by confrontation of plants with bacterial volatiles is probably similar to the effect of plant-pathogen interaction involving direct contact. So the previously reported (a)biotic stress associated with hydrogen peroxide accumulation was investigated by using diaminobenzidine (DAB) staining (Thordal-Christensen et al. 1997). High concentrations of hydrogen peroxide in the cotyledons could be detected after 2 or 3 days in dual cultures with *S. plymuthica* HRO-C48 and *S. maltophilia* R3089, respectively. The one-day delay in the response to *S. maltophilia* R3089 went along with differences in kinetics of growth impairment (Wenke et al. 2012a). This kinetic relationship, the lack of inhibitory effects under coculturing for less than 36 h, and the fact that a high formation of reactive oxygen species under stress conditions has an extremely detrimental effect on cells suggest that impairment of growth and cell death are closely linked to formation of hydrogen peroxide. It should also be taken into consideration that hydrogen peroxide is considered a nonspecific signaling molecule in stress situations. Its formation has been described as one of the initial responses of pathogen defense (Lamp and Dixon 1997) and to many abiotic stimuli as well (Neill et al. 2002; Mittler et al. 2004). Therefore, potentially specific signals can be expected earlier than 2 or 3 days in dual cultures, and later effects (formation of hydrogen peroxide, inhibition and killing of seedlings) can be interpreted as a nonspecific result of volatile exposure. At present it remains speculative to what

extent this hydrogen peroxide formation reflects a similarity to biotic or abiotic forms of stress.

### *16.4.2 Specific Changes in Gene Expression*

Current data show that bacterial volatiles cause death of *A. thaliana* seedlings in dual culture within 5 days. This is accompanied by a marked impairment of leaf and root growth and a systemic accumulation of hydrogen peroxide after 2–3 days. None of these were observed when the seedlings were exposed to volatiles from *S. plymuthica* for a maximum of 1.5 days. It is, therefore, concluded that an exposure time of less than 36 h is especially suitable for analysis of gene expression in order to detect any specific plant responses not solely linked to the dying process.

First indications of transcriptional changes in dual cultures came from established lines of *A. thaliana* in which stress- and pathogen-induced promoter elements control the *uidA* (β-glucuronidase, GUS) gene (Rushton et al. 2002). In the course of these experiments, GUS activity in the cotyledons was detected histochemically using 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) and quantified fluorometrically using 4-methylumbelliferyl-β-D-glucuronide (MUG). Two transgenic lines of plants led to the decisive discovery of lines containing (1) an S box and (2) a Gst1 box. The S box is involved in the regulation of gene expression in response to fungal elicitors in parsley (*Petroselinum crispum*) (Kirsch et al. 2001) and apparently plays a special role in nonhost interaction with pathogens (Rushton et al. 2002). The S box is apparently regulated by APETELA 2/ethylene-responsive element-binding ERF (AP2/ERF) transcription factors (Rushton et al. 2002). In dual cultures with *S. plymuthica* HRO-C48 as well as with *S. maltophilia* R3089, the S box exhibits a volatile-dependent induction within 18 h, as determined histochemically and spectrophotometrically (Wenke et al. 2012b).

During the same time period, the specific activation of the Gst1 box in response to both mixtures of volatiles was detected (Wenke et al. 2012a). This element is already well known as the *gst1* gene of the potato (Strittmatter et al. 1996) and contains both S box and W box. So the Gst1 element is involved in responses to pathogens and in senescence (aging) processes. It is also regulated, in addition to the AP2/ERF proteins, by WRKYs. In conclusion, there is a new realization that WRKY- and AP2/ERF-regulated signaling pathways are activated in less than 18 h, without kinetic differences in the responses to *S. plymuthica* HRO-C48 and *S. maltophilia* R3089.

In order to gain more detailed knowledge of underlying signaling processes, early changes in *A. thaliana* wild-type seedlings at the transcriptome level were analyzed after 6, 12, and 24 h by microarray analysis. Responses to volatile mixtures from *S. plymuthica* HRO-C48 and *S. maltophilia* R3089 were analyzed independently by using ATH1 gene chips (Hennig et al. 2003). Many individuals from five dual cultures each were combined to form a biological replicate; this resulted in well reproducible data with duplicates, which were verified by real-time

PCR analysis as biologically independent on the basis of selected marker genes (Wenke et al. 2012a).

In response to volatiles of *S. plymuthica* HRO-C48 and *S. maltophilia* R3089, either 889 or 655 genes, respectively, underwent an at least twofold transcriptional change compared to the control (Wenke et al. 2012a). In both experiments, considerably more genes were repressed (turned off) than were expressed (turned on). On the other hand, the transcriptome changes induced by both bacteria had kinetically opposing effects. Whereas the volatiles of *S. plymuthica* HRO-C48 induced regulatory responses in most genes after 24 h, much of the response to cocultivation with *S. maltophilia* R3089 could be detected within hours. These kinetics are diametrically opposed to later, perhaps indirect, effects. This supports the assumption that early volatile-induced responses of *A. thaliana* are specifically adapted to the various elicitors and also need to be looked at separately from nonspecific effectors.

A direct comparison (Venn diagram) of the list of genes regulated by both treatments revealed that 162 genes were changed by *S. plymuthica* HRO-C48 and *S. maltophilia* R3089 volatiles at the expression level throughout the whole experiment (Wenke et al. 2012a). The remaining 727 or 493 genes were regulated specifically by volatiles of *S. plymuthica* HRO-C48 or *S. maltophilia* R3089, respectively. It can be assumed that the 162 genes independent of the bacterial species contain, among others, signaling elements responsible for growth inhibition of the plants at a later time. Transcription factor activity is located on 21 of these 162 genes, including three APETELA-2 proteins, six MYB factors, and WRKY18. It would be interesting to hypothesize whether members of these three protein families directly interact with each other in response to rhizobacterial volatiles.

MapMan (Thimm et al. 2004) is a good software tool for summary visualizations of functions of regulated genes. Both data sets of *S. plymuthica* HRO-C48 and *S. maltophilia* R3089 volatile-specific regulated genes were presented in an "Overview of biological stress responses" (Wenke et al. 2012a). A strong involvement of typical responses to pathogens can easily be recognized in dual cultures with *S. plymuthica* HRO-C48. Receptors involved include At5g45070 and At1g65390, which are essential for pathogen defense and the immune system of plants (e.g., Meyers et al. 2002). Also involved are peroxidases, glutathione-S-transferases, and enzymes of secondary metabolism and hormonal signaling pathways (salicylic acid, SA; abscisic acid, ABA; auxin; ethylene), as well as pathogenesis-related (PR) genes, including seven members of the Toll/interleukin1 receptor/nucleotide binding site/leucine-rich repeat (TIR-NBS-LRR) class of proteins, which are essential for pathogen recognition (Dangl and Jones 2001). Considerably, fewer pathogen-response-associated genes were regulated in response to *S. maltophilia* R3089 volatiles, although four proteins of the TIR–NBS–LRR class were involved. Other common features of the specific response to both volatiles were changes in proteolytic processes and cell wall metabolism as possible mechanical defense mechanisms. In addition, several representatives of the family of pathogen defense-associated transcription factors seemed to play a bacterial-nonspecific role in mediating volatile-induced responses: ERF, basic leucine zipper (bZIP),

MYB, and WRKY. Once again, the transcriptional changes of the cell wall stood out in the MapMan visualization of the "metabolic overview" (Wenke et al. 2012a). At the same time, the samples treated with *S. maltophilia* R3089 revealed an inactivation of genes coding for components of the mitochondrial electron transport chain and the photosystems. In part, these may be the cause of the one-day delay of hydrogen peroxide accumulation in response to *S. maltophilia* R3089 volatiles in comparison to the response induced by *S. plymuthica* HRO-C48.

In order to verify a correlation with certain functional activities, 727 or 493 of the volatile-specific genes and the 162 jointly regulated genes were assigned to functional categories according to the gene ontology (GO) annotation of TAIR (Swarbreck et al. 2008; Berardini et al. 2004) (Wenke et al. 2012a). In the set of *S. plymuthica* HRO-C48 specific genes, the categories "DNA, RNA, and protein metabolism" and "cell organization and biogenesis" were significantly underrepresented, also genes with functional roles in mitochondria and ribosomes, in the cytoplasm, and at the plasma membrane. On the other hand, there were significantly more genes of the "transcription" categories and genes involved in general stress responses and in extracellular processes.

With regard to the GO categories, the specific responses to *S. plymuthica* HRO-C48 had little in common with those induced by *S. maltophilia* R3089. The *S. maltophilia* R3089 specific genes had a higher incidence in the categories "cell organization and biogenesis," "mitochondria," and "cytosol." However, the categories "transport," "kinase activity," "nucleic acid binding," "transferase activity," and "developmental processes" are definitely overrepresented. On the basis of the functional categories, the differences revealed between the two responses investigated suggest the involvement of different effectors in the volatile mixtures, which trigger the specific responses in *A. thaliana* within 24 h.

When we consider the functional classification of the 162 genes that respond nonspecifically, it seems that general stress responses as well as transcription factors (TF) are increasingly regulated. In fact, 21 TF that are quite significantly overrepresented ($p \leq 1.3 \times 10^{-42}$) were identified. Some of these proteins have been described as having functions in developmental processes (e.g., two BTB domain scaffold proteins, BT2 and BT4: Robert et al. 2009) or in stress responses (e.g., C2H2-type ZAT10 family protein or WRKY18: Sakamoto et al. 2000; Rossel et al. 2007; Wang et al. 2008). On the other hand, genes of the "transport" and "protein metabolism" groups were significantly less regulated on average.

In order to better assess the type of plant response to rhizobacterial volatiles, the data sets were compared with published microarray data from biotic and abiotic stress experiments, hormone treatments, and the response to growth-enhancing GB03 volatiles (Kilian et al. 2007; Goda et al. 2008; Wanke et al. 2009; Zhang et al. 2007). Surprisingly, this resulted in a relatively uniform picture for the 727 and 493 nonspecific genes and the 162 specific genes (Wenke et al. 2012a). All three data sets revealed a slight overlap with respect to various biotic stresses. As already revealed by MapMan, it was confirmed that the responses to *S. plymuthica* HRO-C48 and to pathogens and the so-called gene-to-gene resistance have somewhat more in common. As for the transcriptional regulation of hormonal

signaling pathways, only abscisic acid and methyl jasmonate are of importance in the nonspecific as well as specific response.

Of all three sets of data, the closest similarity appeared in the responses to abiotic stress with the highest hypergeometric probabilities. Especially those genes were involved that are regulated by cold, osmotic, and salt stress as well as UV-B radiation. Then again, there proved to be very little in common with the dual cultures with *B. subtilis* GB03. This underlines the specificity of changes in plants as an adaptation to volatiles of various bacterial species. The low degree of similarity to oxidative stress was also interesting. This supports the notion that the systematic accumulation of hydrogen peroxide is not a typical response to reactive oxygen species, which includes the classic programmed cell death and appropriate signaling pathways upstream, as is known for defense responses upon pathogen challenge (Neill et al. 2002; Desikan et al. 1998).

The results to date suggest that key factors are present in the 162 generally regulated genes in response to two different mixtures of volatiles, those for inhibition of growth and for chlorosis. Due to the large number of transcription factors (TF) of these 162 genes, many other nonspecifically responding genes might be regulated by these TF. Analysis of *cis*-regulatory elements using the Athena database (O'Connor et al. 2005) revealed that 12 TF-binding motifs are highly enriched ($p \leq 10^{-3}$) in promoters of the 162 genes (Wenke et al. 2012a). Except for the TATA-box motif, all of the elements of stress responses are involved, especially those of biotic and abiotic stimuli, abscisic acid signaling, and light stress. The W-box motif TTGACY is of special interest. It was present in 124 of the 162 genes and also in the GST1-box, which had previously led to a highly volatile-dependent GUS activity in the promoter-GUS test. Moreover, W-box-WRKY interaction is known to play an essential role in important plant processes (Rushton et al. 2010).

### *16.4.3 Involvement of WRKY Transcription Factors in the Mediation of Volatile-Induced Changes*

The higher frequency of the W box in the 162 nonspecifically regulated genes and its presence in the volatile-activated GST1 box directed more attention toward testing of W-box-WRKY interaction. Only AtWRKY18 (At4g31800) was induced to a considerable degree in both volatile treatments (Wenke et al. 2012a). AtWRKY18 has been described as a signal in pathogen defense (Pandey et al. 2010). It belongs to the IIa group of WRKY-proteins and is functionally redundant with its *Arabidopsis* paralogs AtWRKY40 and AtWRKY60 (Xu et al. 2006; Shen et al. 2007; Mangelsen et al. 2008). AtWRKY40 (At1g80840) was also induced in response to *S. plymuthica* HRO-C48 volatiles (Wenke et al. 2012a). WRKY40, among other things, is involved in the regulation of RRTF1 (redox responsive transcription factor) and JAZ8 (jasmonate ZIM-domain), both AP2 proteins (Pandey et al. 2010). Neither RRTF1 nor JAZ8 were regulated in the dual cultures.

To test the hypothesis that WRKY transcription factors are involved in volatile-mediated effects, *WRKY18*, *WRKY40,* and *WRKY60* single mutants were tested in tricultures with *S. plymuthica* HRO-C48 or *S. maltophilia* R3089 and wild-type seedlings of *A. thaliana*. Only the response of *WRKY18* mutants to volatiles of both bacterial species was significantly weaker compared to the wild type. These mutants had almost twice the fresh weight and significantly more total chlorophyll after 3 days in triculture. After 10 days, the relative fresh weight was increased by 300 % (Wenke et al. 2012a). Nevertheless, these *WRKY18* mutants were not capable of surviving. After 10 days they also became extremely chlorotic. DAB assays revealed no clear differences in hydrogen peroxide accumulation between the mutant and the wild type. This supports the hypothesis that seedling death is causally linked to chlorosis but may be indirectly related to accumulation of reactive oxygen species. Furthermore, it can be seen that the volatile-induced responses of *Arabidopsis* include an early WRKY18-dependent signaling pathway but also a later WRKY18-independent pathway involving hydrogen peroxide. With respect to fresh weight, chlorophyll content, and hydrogen peroxide content, both *WRKY40* and *WRKY60* mutants had no significant phenotypic changes compared to the wild type in tricultures with *S. plymuthica* HRO-C48 and *S. maltophilia* R3089.

WRKY18/40 plays an antagonistic role in ABA-dependent signal transduction (Chen et al. 2010; Shang et al. 2010), and WRKY18 positively regulates the JA signals (Wang et al. 2008; Pandey et al. 2010). A comparison of the volatile-regulated data sets with hormonal treatments revealed a large overlap of the ABA and JA effects. However, none of the known ABA or MeJA marker genes were changed in the dual cultures with *S. plymuthica* HRO-C48 or *S. maltophilia* R3089. It remains to be seen whether the ABA- and JA-dependent genes are direct target genes of WRKY18. Pandey and colleagues (2010) reported that the expression of NPR1 (non-expressor of PR genes), the SA marker, remains unchanged in the *WRKY18/40* double mutant. This goes along with the fact that both mixtures of volatiles caused no transcriptional changes in NPR1 or other SA marker genes (Wenke et al. 2012a).

In order to identify potential candidates for the WRKY18-dependent signaling cascade, a comparison was made of all volatile-dependent genes with the 165 genes that are deregulated in the *WRKY18/40* double mutant (Pandey et al. 2010). It turned out that there is an overlap of 70 genes (Wenke et al. 2012a). Of these 70 genes, 41 belong to the *S. plymuthica* HRO-C48 specific data set ($p \leq 1.4 \times 10^{-39}$), 10 to the *S. maltophilia* R3089 specific data set ($p \leq 3.0 \times 10^{-11}$), and the remaining 19 to the 162 genes regulated independent of the bacterial species. In turn, 10 of the 41 genes of the *S. plymuthica* HRO-C48 response are involved in the ethylene signaling pathway: ethylene biosynthesis (1-aminocyclopropane-1-carboxylic acid synthase 6), signal transduction (mitogen-activated protein kinase 9), and signal integration (5 ERFs). Participation of ERFs could already be assumed, based on the volatile-dependent activation of S and GST1 boxes. This was confirmed by transcriptome analysis of cocultures with *S. plymuthica* HRO-C48. In addition, 54 W boxes were located in the 19 genes that reacted to the volatiles in a bacterial-nonspecific manner and to the wrky18/40 double mutation, which means an average of 2.8 W boxes per promoter. This value leaves open the question whether

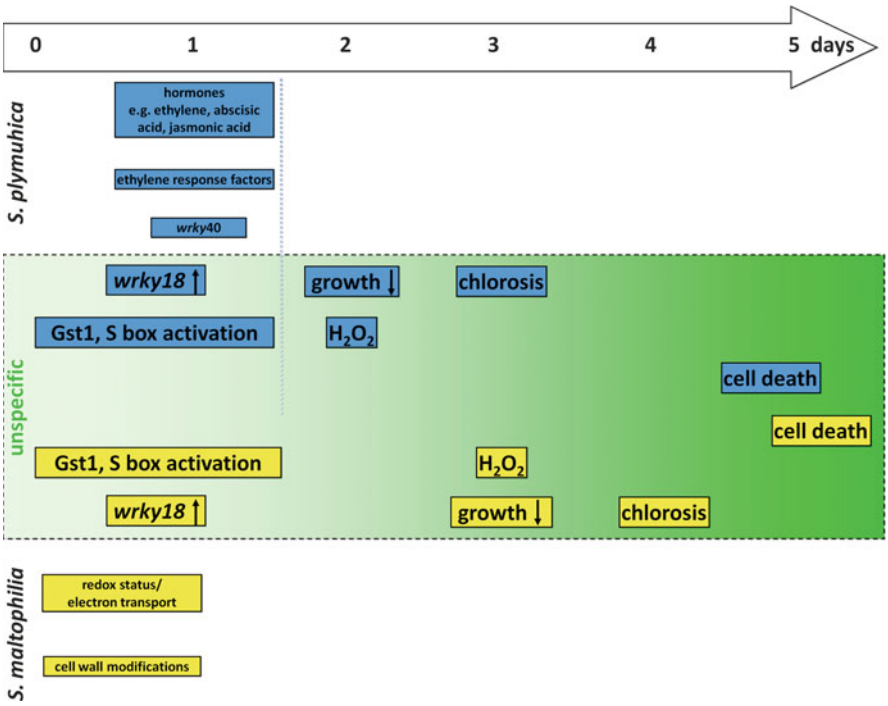


**Fig. 16.1** Overview of transcriptional and morphological as well as physiological alterations in *Arabidopsis thaliana* in response to rhizobacterial volatiles

the 19 genes are under the direct control of WRKY18 and/or WRKY40. Aside from WRKY18, there are eight other TF among the 19 genes: two AP2/ERF, two MYB factors, two BT proteins (BTB and TAZ domains), SZF1 (salt-tolerance zinc finger 1), and ZAT10 (zinc finger, C2H2 type). These all play a role in stress responses and/or in hormone signal transduction.

A time schedule of events that occur in *A. thaliana* at the morphological, physiological, and transcriptional level in dual culture assays with both rhizobacterial strains is summarized in Fig. 16.1.

The observations are divided into strain-specific (in the white/green box in the middle) and strain-unspecific effects as well as into *S. plymuthica* (blue boxes) and *S. maltophilia* (yellow boxes) volatile-induced effects. The gray, dotted line represents the time of no return for the response to the *S. plymuthica* volatiles.

## 16.5 Which Type of Stress Is Induced by Rhizobacterial Volatiles?

The triggering of specific plant responses to airborne substances is one aspect of plant-pathogen interaction. That largely has not been taken into account. Biotic, as well as abiotic, exogenous or endogenous elicitors (pathogen- or microbe-associated

molecular patterns PAMPs/MAMPs, effectors, and damage-associated molecular patterns: DAMPs) initiate in plants a number of finely balanced and coordinated mechanisms. These elicit local or systemic resistance to pathogens or wound-healing responses following mechanical stress (summarized in, e.g., Chisholm et al. 2006; Lotze et al. 2007; Boller and Felix 2009). Plants have various means of signal transduction to elicit such specific responses: ionic currents, formation of reactive oxygen species, mitogen-activated protein kinase cascades, hormones (SA, JA, ET), other protein kinases, and phosphatases (summarized in Hématy et al. 2009). Up to now, the potential of volatile-induced specific responses had not been taken into account with regard to signal transduction under stress conditions. Therefore, a new term for volatile interspecies-active elicitors should be introduced at this point: "microbial volatile-associated molecular patterns" (mVAMPs).

This work focuses on the effects of a mixture of a number of potentially active volatiles. It could be shown with two rhizobacterial species that different volatile mixtures are capable of initiating specific responses in *A. thaliana* at an early point in time. These responses revealed factors such as WRKY18 that play an important role in the classical response to pathogens. An overall comparison of the various stress transcriptomes of *Arabidopsis* revealed that the two responses studied in dual cultures are more similar to abiotic stress responses than to those of pathogen defense. The involvement of several classical transducers of pathogen defense, in particular with regard to the transcription factors, demonstrates that a considerable number of PAMP responses up to now may have been induced by volatile metabolites. On the other hand, the absence of essential PAMP- and DAMP-regulated genes makes clear that mVAMP-induced stress is a new type of stress.

On the basis of present data, a model was developed that summarizes the new findings on the responses of plants to bacterial volatiles (Wenke et al. 2012a). Within the scope of this work, apparently general as well as bacterial species-specific mVAMP-induced changes in the gene expression of *A. thaliana* in dual culture with *S. plymuthica* HRO-C48 or *S. maltophilia* R3089 were discovered. The bacterial species-specific responses at the transcript level entailed signal transduction via the hormones ethylene, ABA, and JA after 24 h with *S. plymuthica* HRO-C48, whereby the corresponding upstream regulators of the ERF and WRKY40 groups are involved. In contrast, there is the very rapid, specific response to the *S. maltophilia* R3089 volatiles with respect to regulation of the redox potential and the electron transport chain. This was followed by very early transcriptional changes in the cell wall, which may serve to strengthen the mechanical barrier against the volatiles. This specificity is likely a unique characteristic of the differing volatile compositions (Kai et al. 2007).

In both treatments there were various indications that the family of plant-specific WRKY-TF plays an important role. The essential factor is apparently WRKY18, regardless of the type of volatile mixture. This factor has been described in the literature as a negative transcription regulator, which at the same time has an antagonistic effect on expression of the paralog factor WRKY40 (Xu et al. 2006; Shen et al. 2007; Chen et al. 2010; Pandey et al. 2010). *WRKY18* mutants had a significantly weaker inhibited phenotype in direct comparison with the wild type.

Therefore, the hypothesis of a double-negative cascade can be proposed. A yet unknown gene is repressed via WRKY18. The inactivated target gene itself codes directly or indirectly for an inactivator of cell death. In *WRKY18* mutants, this can extend the viable phase of the seedlings, since the WRKY18-dependent inactivation of the cell death repressor has been turned off. This makes it possible for such mutants to grow significantly better under cocultivation. As to the accumulation of hydrogen peroxide, it remained unchanged in the *WRKY18* mutants, but in the wild type in dual cultures with *S. plymuthica* HRO-C48 or *S. maltophilia* R3089, there were kinetic differences. These in turn can be explained by bacterial species-specific responses. These facts as a whole suggest a specific stimulation of hydrogen peroxide formation leading to cell death, independent of WRKY18.

This study on the effects of two different mixtures of rhizobacterial volatile metabolites has revealed a new group of stressors (elicitors), the mVAMPs (microbial volatile-associated molecular patterns). The responses of plants to these mVAMPs include general as well as bacterial strain-specific changes. At the same time, the regulation of classical stress marker genes described previously for stress situations (MAMP, PAMP, DAMP dependent) was not detected, which underlines the novelty of the mVAMP-dependent stress situation.

## 16.6 Potentially Biologically Active Individual Substances

Despite the important differences found using gene expression data, cocultivation with *S. plymuthica* HRO-C48 as well as *S. maltophilia* R3089 causes a considerable impairment of plant development accompanied by complete chlorosis and a systemic hydrogen peroxide accumulation leading to systemic cell death. It can, therefore, be concluded that identical or very similar single compounds are responsible for these nonspecific changes. The identification of single active substances, including their effective concentrations in dual cultures, is very complicated and difficult. In order to identify potentially active substances in the Petri-dish setups, known and accessible compounds were tested that might play an important role in cocultures with *S. plymuthica* HRO-C48 and *S. maltophilia* R3089, including 2-phenylethanol, dimethyl disulfide (DMDS), HCN, and $NH_3$. Plants as well as bacterial and fungal microorganisms emit 2-phenylethanol, the odor of roses.

This compound has antimicrobial properties because of its ability to alter plasma membrane permeability as well as amino acid and sugar transport (Etschmann et al. 2002). In dual cultures, 20 µmol 2-phenylethanol proved capable of inhibiting *A. thaliana* growth by 50 % (Wenke et al. 2012a). It has been seen that another bacterial volatile, dimethyl disulfide, has a similar affect in dual cultures (Kai et al. 2010). In addition, dimethyl disulfide has insecticidal properties due to its ability to inhibit cytochrome oxidase of the mitochondrial electron transport chain and the potassium channel (Dugravot et al. 2003; Gautier et al. 2008). HCN is a much-discussed compound in connection with volatile-induced inhibition of plants. It is produced by *Pseudomonas*, *Chromobacterium*, and *Rhizobium* (Blumer and Haas

2000; Kai et al. 2010; Blom et al. 2011). Even 1 μmol HCN causes a 400 % decrease in fresh mass (Blom et al. 2011). In several studies with noncyanogenic wild-type strains or HCN-negative mutants and HCN-producing bacteria, a correlation could be established between HCN production and inhibition of plant growth (fresh mass, root length) (Blumer and Haas 2000; Blom et al. 2011; Wenke et al. 2012b). During the studies of Blom and colleagues, it was shown that *Serratia* species are not capable of producing/releasing hydrogen cyanide, similar to that of *S. odorifera* 4Rx13 (Kai et al. 2010). They demonstrated that neither *S. plymuthica* HRO-C48 nor *S. maltophilia* R3089 emit HCN in Petri dishes on NB medium (Marco Kai unpublished). On the other hand, the emission of ammonia was detected in both strains (Teresa Weise unpublished), similar to the $NH_3$ concentrations released by *S. odorifera* 4Rx13 (<1 μmol, Kai et al. 2010). It is known that ammonia causes decoupling of electron transport (Losada and Arnon 1963), which leads to chlorosis and complete inhibition of plant growth (Britto and Kronzucker 2002). Since at least 2.5 μmol of ammonia is required to cause a distinct inhibition of *A. thaliana* in dual culture (Kai et al. 2010), it can be assumed that ammonia is not solely responsible for volatile-dependent effects on *A. thaliana* in coculture with *S. plymuthica* HRO-C48 and *S. maltophilia* R3089, but has the potential to act synergistically. Based on previous findings, it is concluded that ammonia, dimethyl disulfide, and 2-phenylethanol have a potentially additive or synergistic effect on plants. Additional testing of single compounds or mixtures of these in varying proportions is required to gain more precise information.

## 16.7 Ecological Aspects of Volatile-Induced Effects

The importance of these effects for the ecosystem should be discussed briefly at this point. In fact, it should be resolved to what extent these effects are applicable to natural conditions, despite the simplicity of the testing system. The conditions chosen proved effective in complete killing the seedlings. The cultivation parameters, in particular the supply of nutrients available to the bacteria, corresponded closely to ideal conditions and resulted in bacterial cell counts of up to $10^{11}$ cfu. A quantification of *S. plymuthica* HRO-C48 as well as *S. maltophilia* R3089 cells in dual cultures in the presence or absence of *A. thaliana* showed that the seedlings had no significant effect on the rhizobacteria via the air space (unpublished). However, there have been several reports that root exudates are effective against microorganisms, so plants are capable of defending themselves directly (summarized in Bais et al. 2006). This type of interaction via soluble compounds was prevented by spatial separation in Petri dish assay used here. Under these artificial conditions, interaction is not only unilateral but also limited to only two interaction partners. In natural surroundings, a balance within the rhizosphere community would be achieved by intraspecies and interspecies competition. New experiments with various combinations of bacteria in plant cell cultures

have verified that the greater the species diversity, the more stable the microbial community under stress situations (Chatzinotas et al. 2011).

Another aspect for consideration is the type of stress application. The effective volatiles are wafted toward the plants via the airspace above the surface, which does not resemble the natural situation. As for the mechanism of stress recognition, it remains uncertain the involvement of receptors and which plant organs actually detect the volatile signals in dual cultures. This indicates that the simplified experiments carried out here must be followed by performing similar experiment in natural surroundings (spatial separation in soil) in order to obtain ecologically relevant information. Volatile metabolites play an ecological role in the transmission of information under natural conditions. For example, field trials have demonstrated indirect resistance in maize, which was capable of recruiting entomopathogenic nematodes by β-caryophyllene emission (Rasman et al. 2005). In conclusion, the present study provides a sound basis for further studies to shed light on the obvious potential of volatile substances as elicitors of specific responses of plants from an ecological viewpoint.

In addition to artificially induced genetic changes, the occurrence of natural genotypic and phenotypic variants within a species provides an invaluable source for studying complex responses to ever-changing conditions. More than 750 accessions have been described for *A. thaliana*. A comparison of transcriptomes altered by volatiles with the expression profiles of a wide variety of *A. thaliana* accessions under normal conditions revealed the ecotype-specific expression of a large portion of the volatile-regulated genes. This means that the accessions bring along different initial conditions for responding to volatile metabolites at the transcript level. Based on these insights, a number of accessions were tested in dual cultures with *S. plymuthica* HRO-C48 and *S. maltophilia* R3089. Aboveground fresh biomass and root development of 21 natural variants of *A. thaliana* under microarray conditions with *S. plymuthica* HRO-C48 were determined (Wenke et al. 2012b). With regard to the aerial parts of all *Arabidopsis* variants selected, there were no significant differences in relative growth inhibition by the volatiles (Wenke et al. 2012a). Relative inhibition was around 90 % in all accessions in comparison to untreated controls. Such a high percentage of inhibition implies that the seedlings were killed quickly. According to previous findings, chlorosis and killing of plants may be a nonspecific late effect of volatiles, which should be considered separately from specific immediate responses. With regard to the roots, accession-specific responses to volatiles of *S. plymuthica* HRO-C48 were observed. The greatest variation was found between C24 (82 % inhibition) and Ler (42 % inhibition). An adaptation to L-glutamate was confirmed by similar values for relative inhibition of primary root growth of C24 and Ler (approximately 80 % and 40 %, respectively) (Walch-Liu et al. 2006). The natural surroundings of C24 are unknown. It is assumed that this variant originated in the laboratory and has, therefore, not undergone natural adaptation to rhizobacterial volatiles. Ler is an ecotype from Landsberg, Germany, which may be adapted to the effects of volatile elicitors. This initial data supports the notion that the discussed specificity of volatile-induced changes in plant processes has an ecological background.

## 16.8 Concluding Remarks and Future Perspectives

Volatile signals allow an intra- and interspecies exchange of information between organisms without direct contact, also beneath the soil surface. The rhizobacteria *S. plymuthica* and *S. maltophilia* emit quite different mixtures of volatiles that cause enormous transcriptional, physiological, and morphological changes in *A. thaliana*. These in turn lead to seedling death within 5 days. Research on bacterial volatiles is still in its infancy. It will remain an exciting topic in the coming years: identification of yet unknown infochemicals and in-depth elucidation of their potential as important pharmaceutical, ecological, and agricultural effectors. This includes not only elucidation of the biosynthesis of volatile metabolites but also decryption of volatile-induced signaling pathways in interaction partners.

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