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Cyclic monoterpene mediated modulations of *Arabidopsis thaliana* phenotype

Effects on the cytoskeleton and on the expression of selected genes

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Monoterpenes at high atmospheric concentrations are strong growth inhibitors in allelopathic interactions. Effects depend on dose, molecular structure of the monoterpene and on the species of the receiver plant. Stomata are among the first targets affected by camphor and menthol. Previously, it could be demonstrated that the compounds induce swelling of the protoplasts, prevent stomatal closure and enhance transpiration. In this study, we show that the block of stomatal closure is accompanied by changes to the cytoskeleton, which has a direct role in stomatal movements. Although *MPK3* (MAP3 kinase) and *ABF4* gene expressions are induced within six hours, stomatal closure is prevented. In contrast to *ABF4*, *ABF2* (both transcription factors) is not induced. *MPK3* and *ABF4* both encode for proteins involved in the process of stomatal closure. The expression of PEPCase, an enzyme important for stomatal opening, is downregulated. The leaves develop stress symptoms, mirrored by transient changes in the expression profile of additional genes: lipoxygenase 2 (*LOX2*), *CER5*, *CER6* (both important for wax production) and *RD29B* (an ABA inducible stress protein). Non-invasive methods showed a fast response of the plant to camphor fumigations both in a rapid decrease of the quantum yield and in the relative growth rate. Repeated exposures to the monoterpenes resulted finally in growth reduction and a stress related change in the phenotype. It is proposed that high concentrations or repeated exposure to monoterpenes led to irreversible damages, whereas low concentrations or short-term fumigations may have the potential to strengthen the plant fitness.

Introduction

Monoterpenoids are constituents of the VOC-(volatile organic compound) bouquets emitted by plants. Many of these compounds have been identified as mediator molecules in plant-herbivore, plant-microorganism and plant-plant communication. Following adsorption of VOCs at the leaf surface, uptake into the leaf can occur via the stomata or by cuticle diffusion.[1] Analysis of the molecular backgrounds of the defense mechanisms that take part in plant-insect, plant-microbe, plant-herbivore interactions has become an important topic during the last two decades.[1-5] VOCs trigger the release of volatile terpenoids in tomato plants and induce defence genes in Arabidopsis.[6] Lima beans, exposed to terpenoids, responded in a similar manner.[7] Other roles in plant-plant interactions have received less attention, although a high atmospheric abundance of monoterpenoids is known to have essential influences on structuring distribution, density and diversity of species in different plant communities, such as the High Chaparral in south California or the sand pine scrub communities in Florida. In these communities, the compounds suppress the growth of individuals of other or of their own species.[8-11]

On the other hand, monoterpenes have been shown to protect leaf membranes from oxidation and to increase heat stress resistance by modification of the leaf thermal tolerance.[12,13] It was assumed that the release of monoterpenes present in low atmospheric concentration in Mediterranean canopies enhanced heat stress resistance of other plants of the community.

Thus, depending on their chemical structure and the dose, monoterpenes can have different effects. Whereas α-pinene protects the photosynthetic apparatus, α-terpinol is even toxic under non-heat stress conditions. Exogenous monoterpenes in high concentrations of 0.5 g/l are toxic to plant cell cultures.[14] Zunino and Zygadlo[15] reported on oxidative stress induction and lipid oxidation induced by the monoterpenes 1,8-cineole, thymol, geraniol, menthol and camphor in maize roots. Essential oil of *Artemisia scoparia* with β-myrcene, limonene, β-ocimene and γ-terpinene

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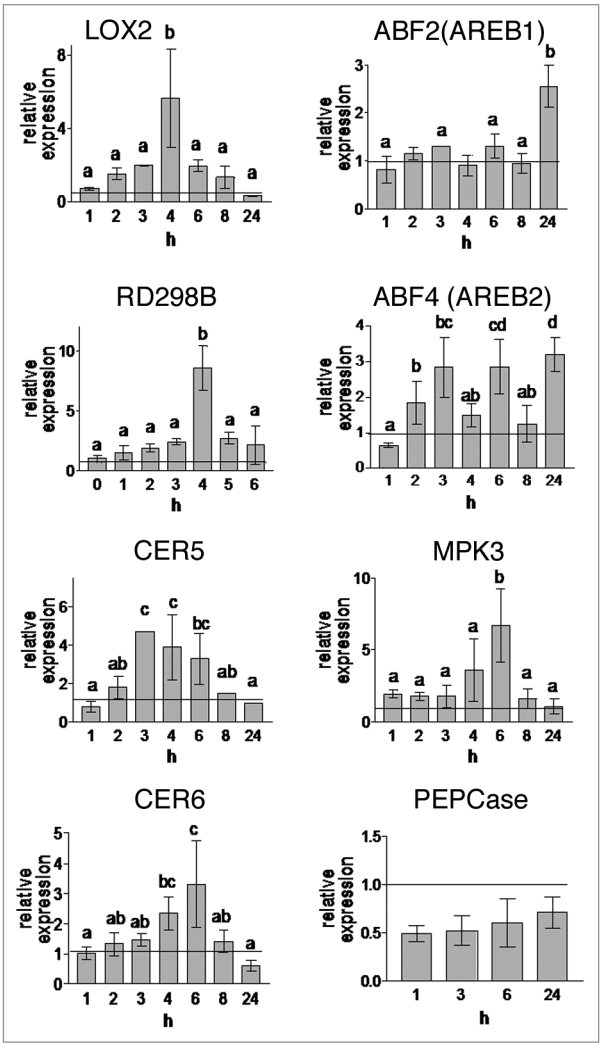

Figure 1. Effects of the monoterpenes on gene expressions analyzed by qRT-PCR. The abundance of mRNA (x fold) is shown relative to the control values referred as to 1 (line). Mean values ± SD of three independent experiments. Letters above the histogram bars refer to statistically significant differences ($p < 0.05$) within groups as determined by Duncan test. Values marked by the same or no letter are not significantly different within bar values.

as major compounds generates ROS and oxidative damage in receiver plants.[16] Peppermint essential oil caused a decrease in membrane potential hyperpolarization at 5 to 50 ppm, but 100 to 500 ppm increased depolarization. The induced membrane depolarizations are known to change ion fluxes across the plasma membrane. The increase in membrane polarization was also found to be correlated to a decline in water solubility of several monoterpenes tested. In cucumber roots, menthol induced an increase of cytosolic free calcium ions, an event that may trigger many signal transduction pathways.[17] Monoterpenes increase the production of phenolic compounds in cell cultures of *Pelargonium fragrans*.[14] A calcium influx and protein phosphorylation/dephosphorylation dependent induction of the expression of PAL (phenylalanine ammonia-lyase: a key enzyme in phenylpropane assembly), PR-2 (pathogenesis related protein-2: β-1,3-glucanase), PR-3 (pathogenesis related protein-3: chitinase) and farnesyl pyrophosphate synthase (FPS) was reported by Arimura et al.[7] The acyclic monoterpenes ocimene and myrcene induced substantial changes in the transcription of several hundred genes in Arabidopsis, many of them are annotated as transcription factors, stress and defence genes.[18] Allo-ocimene is known to prime defence reactions in Arabidopsis against *Botrytis cinerea*, for example by accumulation of antifungal substances and enhanced lignification.[19]

In allelopathic interactions, detailed studies are published for camphor and 1,8-cineol. Both compounds, which are strong growth inhibitors, leading to growth abnormality, inhibition of respiration of isolated mitochondria, aspartate synthase activity and mitosis.[20-23] The present state of knowledge clearly points to a strong dose- and structure-dependency that trigger positive or negative effects of monoterpenoids on defined plant species, cells, tissues and organs. Meanwhile, many studies demonstrate that plant volatiles can have a great future in sustainable development of agriculture. They may be used for pest control, to monitor plant health and to suppress weeds or to modulate plant fitness.

In a previous study, it was demonstrated that the waxy leaf surface and the stomata are among the first targets affected by the cyclic monoterpenes camphor and menthol. The compounds induced stomata opening and swelling of the protoplasts accompanied by the inability of stomata to close as long as the compounds were present. Long term exposures to high concentrations resulted in irreversible desiccation and plant death.[24] Here we show that the block in stomatal closure is accompanied via a change in the cytoskeleton, especially in the actin filaments, which have direct roles in stomata movements.[25-32] Consequently, the leaves develop stress symptoms, mirrored by changes in the expression profile of several selected genes. Repeated exposures to the monoterpenes resulted finally in growth reduction and a changed, stress-related Arabidopsis phenotype.

Results

Modulation of gene expression. Since cyclic monoterpene applications led to opening of stomata, an enhanced transpiration and dehydration, we set out to characterize the expression of several selected genes involved in stomata movement, abiotic stress response (drought, osmotic stress) and wax synthesis by real time PCR.

MAP kinase 3 (MPK3), known to be activated in response to H_2O_2 and ABA, has an important role in guard cell signaling and promotion of stomatal closure.[33] The kinase activity was found to be induced by H_2O_2 and ABA, which both play a key role in adaption to drought and other stresses.[34,35] In our study, the expression of the kinase gene was upregulated 3–6-fold after 4 to 6 h of fumigation (**Fig. 1**). On the other hand, transcription of phosphoenolpyruvate carboxylase (PEPCase), which fixes CO_2 into oxaloacetic acid for malate production, was downregulated. Malate, however, is important for stomatal opening. During the whole period of treatment, *PEPCase* transcript levels were always below the controls. Expression of the ABA-induced genes *ABF2 (AREB1)* and *ABF4 (AREB2)*, encoding basic leucine zipper

transcription factors, were included in this study.[36,37] *ABF4* is suggested to influence directly the regulation of gene expression in guard cells and is known to be involved in drought tolerance and reducing water loss.[38] Abundance of *ABF4* transcripts oscillated, with increases (about 3 fold) after 3, 6 and 24 h. *ABF2* (*AREB1*) was not induced during the first 8 h, but after 24 h, transcript levels were about 2.5 fold. An 8 fold induction of the ABA-inducible and, dehydration responsive *RD29B* gene was observed after 6 h, but the induction was transient. The expression profiles of these few genes indicate a moderate water deficiency in the leaves due to monoterpene fumigation. This is consistent with the induction of *CER5* after 3–6 h and *CER6* after 4 h. Both genes encode proteins essential for the production of the wax layer of plant aerial surfaces.[39,40]

Lipoxygenase 2 catalyzes the hydroperoxidation of fatty acids with cis, cis-1,4-pentadiene structures such as linolenic and linoleic acid.[6,41,42] The products are precursors of C_6-volatiles, whereas hydroperoxylinolenic acid presents an intermediate of jasmonic acid (JA) biosynthesis, a plant growth regulator. *LOX2* expression is regulated in response to stress conditions such as wounding or water deficiency. In our study, this gene was upregulated 2–7-fold after 3–4 h of fumigation. Long term exposure resulted in a downregulation.

Effects on the cytoskeleton. Monoterpenes caused reorganisation and partial disruption of F-actin filaments leading to aberrantly over-polymerized actin cytoskeleton (**Fig. 2A and B**). Typical actin filaments in control stomata showed radial orientation and the cell periphery showed strong signal. However, in monoterpene-treated plants actin arrays were replaced by less-organized F-actin networks and over-polymerized patches. In addition, F-actin was not assembled abundantly at the cell periphery in monoterpene-treated plants. Also microtubules were sensitive to the monoterpenes showing over-polymerized radial arrays whereas they were depleted at the open stomatal pore (**Fig. 2C and D**). Overall, these cytoskeletal reorganizations were fully consistent with the morphological changes observed, such as the swelling of guard cells and opening of stomata. The cytoskeleton is known to integrate sensory signals with ionic activities and metabolic processes.[29,30] The aberrantly organized and perhaps less dynamic cytoskeleton is proposed to be a major reason for the failure of endogenous signals to induce stomatal closure after monoterpene fumigation. Disruption of F-actin filaments had no effect on actin 2 expression.[43]

Modulations of the phenotype. Stress induced modulations occur at multiple spatial and temporal levels and require sensitive phenotyping techniques.[44] Therefore, phenotypic modulations were assayed with GROWSCREEN FLUORO, which captures plant size as projected leaf area (A_{PT}) and integrates chlorophyll fluorescence over the A_{PT}.[45]

Camphor treatment led to a rapid decrease of relative growth rate of the plants (**Fig. 3B**), which was followed by noticeable differences in A_{PT} (**Fig. 3A, Table 1**) of the treated plants compared to the untreated plants. Smaller plant size was accompanied by a lower number of leaves (insert in **Fig. 3A**) Concomitant with the decrease of growth, quantum yield of the leaves was lowered (**Fig. 3C**). Whereas quantum yield recovered after the end of the camphor fumigation, growth rate remained low. A second period of camphor treatment amplified the differences in plant sizes and lowered quantum yield again. Camphor treatment modified plant morphology in terms of higher surface coverage (**Fig. 3D**) and stockiness (**Fig. 3F**) compared to untreated plants. This hints at a more compact growth, i.e., the leaves of the rosette were closer together than in the untreated plants. Especially during the second period of camphor treatment, plants became eccentric (**Fig. 3E**) while the untreated plants were more round-shaped. The eccentricity was due to an unequal distribution of leaves around the centr of the rosette, associated with the formation of lesions during prolonged camphor treatment (**Fig. 4**).

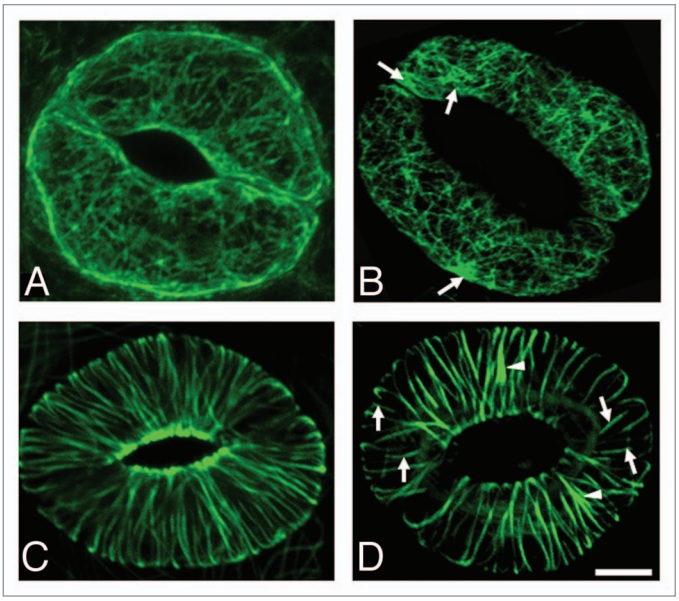

Figure 2. Effect of camphor and menthol on the cytoskeleton in stomata guard cells. Effect of camphor (10 mg/l) and menthol (5 mg/l) on the cytoskeleton in stomata of *Arabidopsis thaliana* cotyledons. Actin cytoskeleton visualized with the *35S::GFP:FABD2* construct as in vivo marker for filamentous actin in control conditions (A) and after treatment with monoterpenoids for 48 h (B). Arrows point to the patches of bundled F-actin. Microtubular cytoskeleton visualized with the *35S::GFP:MBD* construct as in vivo marker for microtubules in control conditions (C) and after treatment with monoterpenoids for 48 h (D). Note pronounced bundling of microtubules (arrowheads) and appearance of short disrupted microtubular bundles (arrows). Bar: 10 μm.

Discussion

Abscisic acid is known as a key inducer of stomatal closure in response to the plant water status. It also mediates responses to various stresses by modifying expression of an assortment of genes.[34,45] Fumigation with camphor and menthol causes water stress due to the inability of stomata to close, with the consequence of high transpiration.[24] Guard cell linked MPK3, required for stomatal closing, is activated by ABA and H_2O_2.[33,47,48] The promotor of *RD29B*, a marker gene of ABA-induced gene expression, contains an ABRE (abscisic acid responsive element). A number of basic leucine zipper transcription factors can bind to ABRE, such

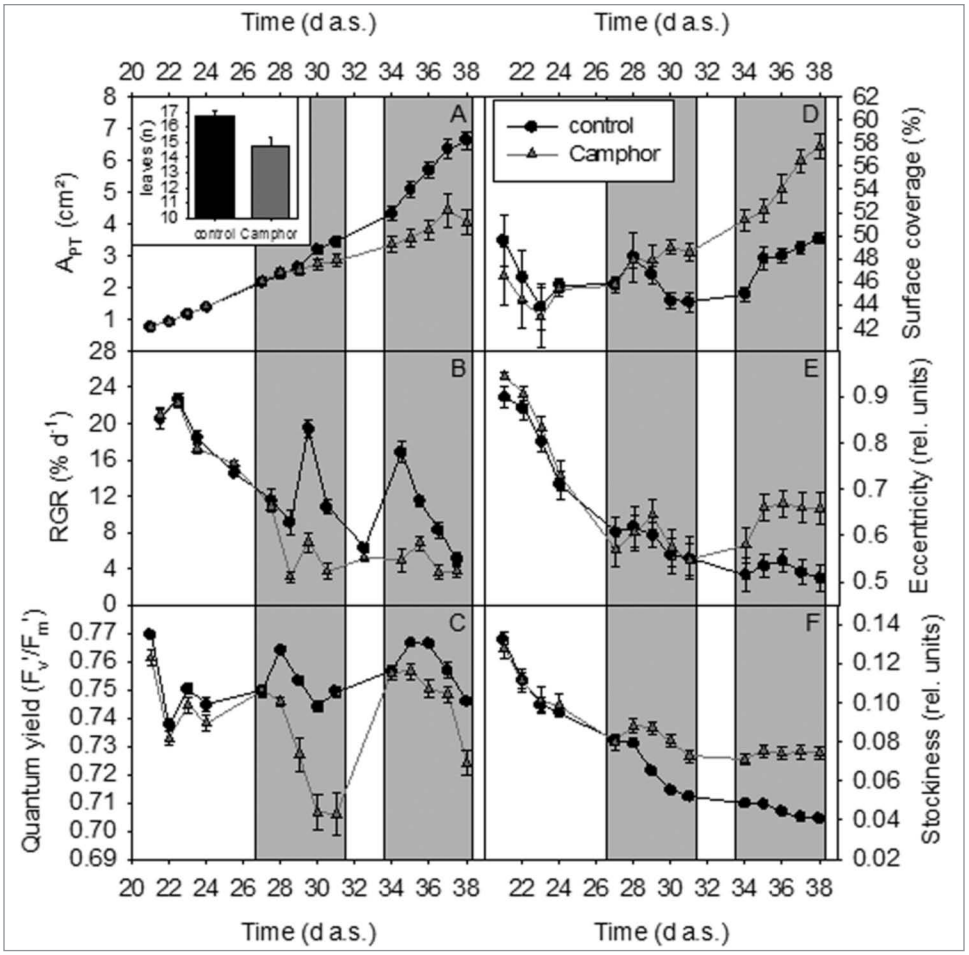

Figure 3. Modification of *A. thaliana* phenotypes analyzed with GROWSCREEN FLUORO. Black lines/bars: control treated plants, grey lines/bars camphor treated plants. (A) projected leaf area (A_{PT}), insert in (A), amount of leaves at the final data acquisition; (B) relative growth rate (RGR); (C) quantum yield; (D) surface coverage; (E) eccentricity; (F) stockiness. Dark background indicates periods of fumigation.

Table 1. Phenotypes influenced by camphor treatment

growth	↓
amount of leaves	↓
surface coverage	↑
stockiness	↑
eccentricity	↑
photosynthetic performance	↓

as ABF4 (AREB2) and ABF2 (AREB1).[36] In response to camphor and menthol, the expression of *MPK3, ABF4* and *RD29 B* was induced within six hours, in contrast to *ABF2*. It has been shown that *ABF2* is important in the regulation of seedling growth and responses to glucose. *ABF4*, among others, is required for ABA, stress responses and stomatal closure.[49,50] PEPCase is a major enzyme in malate synthesis. Malate is known to be an important counterion for potassium, which drives stomatal opening.[51] A downregulation of the *PEPCase* transcript level in response to monoterpene induced high transpiration rates seems reasonable. An osmotic and salt repressed *PEPCase* expression was reported by Ueda et al.[52]

Water stress in monoterpene fumigated Arabidopsis leaves is also indicated by the upregulation of *CER6* and *CER5* transcription. Whereas CER6 is the most important condensing enzyme involved in VLCFA (very long chain fatty acid) production—an early step in wax biosynthesis, *CER5* is responsible for the transport of wax monomers out of epidermal cells to the surface. *CER6* transcript accumulation is known to be enhanced in response to osmotic stress. The presence of ABREs in the *CER6* promotor led to the suggestion that the gene is ABA inducible. Hooker et al.[39] demonstrated a 2.5- to 3-fold enhancement of *CER6* transcripts after treatment with 0.1 mM ABA. Similarly, *CER5* was found to be inducible by 50 μM ABA and osmotic stress (Panikashvili et al. 2007).[53] Both genes were upregulated only 3–5 h after the start of fumigation. A 3.5- to 5-fold increase in *LOX2* transcript abundance within 24 h was recently demonstrated by Godard et al.[18] after treatment of Arabidopsis with the acyclic monoterpene ocimene. *LOX2* is a key enzyme in jasmonate biosynthesis and C_6-volatiles production, proposed precursor molecules for the syntheses are the glycolipids of chloroplasts.[6,54] Induced expression and an increase in *LOX2* activity occurred in lima beans exposed to volatiles of emitter leaves infested with *Tetranychus*

urticae. Thus, induction of *LOX2* gene is possible with a large variety of monoterpenoids. The resulting increase in jasmonate production is known to modulate the expression profiles of many genes, including defense genes, and to prime defense reactions.[7,19] A microarray-based screening of jasmonate-responsive Arabidopsis genes by Jung et al.[55] revealed an upregulation of 74 genes including *LOX2* (6.8-fold after 24 h) whereas 63 genes were repressed.

In this study, the upregulation of all genes investigated was transient. Possibly, a single fumigation period of 24 h was not sufficient to manifest stress physiology and plants recovered or the consequences of the disturbed actin cytoskeleton, together with drought stress reactions, inhibited continued gene inductions. It is rather likely that camphor and menthol led to modulations of many other gene activities, in addition to the few investigated in this study. Antagonistic interactions between ABA and jasmonate signaling pathways have been described by Anderson et al.[56] On the other hand, methyljasmonate is assumed to stimulate ABA production.[57] Hassanein et al.[58] describe ameliorating effects of jasmonic acid in plants under drought stress. Jitratham et al.[59] reported on stomatal closure in citrus leaves by jasmonate applications. We propose therefore that the monoterpenes camphor and menthol directly affect the actin cytoskeleton as well as microtubules, abolishes ABA and JA mediated stomatal closing and prevent actions which enable leaves to cope with drought stress. As a consequence, drought stress symptoms set in some hours after starting the treatment with the monoterpenes. According to first studies, Arabidopsis treated with *Arthemisia camphorata* volatiles shows, for instance, a similar increase of *MPK3* expression (data not shown).

Stabilization of actin filaments by phalloidin treatment is known to inhibit stomatal closing in a concentration-dependent manner, whereas depolymerization and fragmentation of actin filaments by cytochalasin D increases stomata opening.[25] These older data have been confirmed later.[26,27,32] Our data reveal that the actin cytoskeleton is very sensitive to monoterpenes not only in guard cells but also in root cells (data not shown). Presumably, the monoterpenes cause depolarizations of the plasma membrane too, affecting activities of ion channels.[26,29,30] Changed ion fluxes across the plasma membrane and dysfunctional ion channels would enhance the effects on the cytoskeleton, interfering with the processes of stomatal closure and movements. As a consequence, water flux into the leaves does not compensate water loss by transpiration and leaves start to suffer from drought stress, even where plants were well watered. Effects on the actin cytoskeleton in mammals seem to be a common mode of action of several monoterpenes, which inhibit bone resorption. This was observed with osteoclasts from rats.[60] Besides the actin cytoskeleton, microtubules are affected by monoterpenes and they are also implicated in stomatal movements.[31] Dependency of the biological activity of monoterpenes on their chemical structure is ascertained by the work of Chaimovitish et al.[61] who show an immediate reaction of plant cells to citral which disrupts microtubules, whereas actin filaments remain intact. These features are highly consistent with the voluminous reports that the cytoskeleton is both regulator and target of biotic interactions (reviewed in ref. 62).

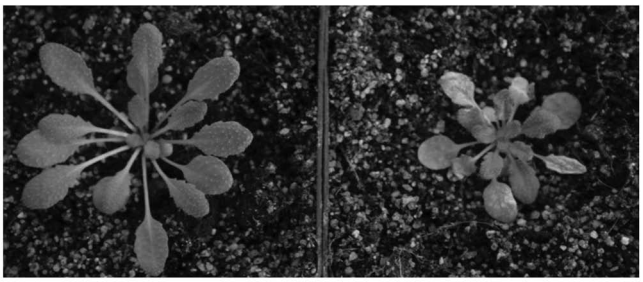

Figure 4. Phenotypes of untreated (left) and camphor-treated plant (right) at the end of the measuring period.

Camphor has the potential to modulate the phenotype of plants. In this study, we showed that repeated fumigation with camphor led to stress-related phenotypic changes of Arabidopsis plants. Lowering of growth and photosynthetic performance demonstrated an adverse effect of the fumigation on plant performance (**Table 1**). The camphor-induced growth phenotypes were similar to those observed under drought stress.[45] Finally, camphor-mediated lesion formation destroyed leaf material and thereby led to eccentric rosette shapes. The increase in compactness and surface coverage of the fumigated plants (**Table 1**) counteracts water loss and can be seen as a response to the high transpiration. Thus, high concentrations, prolonged or repeated exposure to monoterpenes led to irreversible damage of the whole plant, whereas low concentration or short term fumigations with bioactive monoterpenes can lead to reversible responses and may strengthen the plant.

Materials and Methods

Real time PCR. For real time PCR studies 3 week old *Arabidopsis thaliana* ecotype Col-0 plants were placed in plastic boxes and fumigated as described in Schulz et al.[24] for 1, 2, 3, 4, 6, 8 and 24 h with 10 mg camphor/L and 5 mg menthol/L.

Leaves were harvested and used for RNA isolation with the RNeasy Plant Mini Kit (Qiagen) according to the instructions of the manufacturers. Reverse transcription was performed with the Fermentas or with the Quantitec Rev. Transcription kit (Qiagen). cDNA synthesis was performed with DNAse treated RNA (0.2 μg). For real time PCR the POWER SYBR Green PCR Master Kit, microplates LSH 96 well and 3G optical adhesive covers (all from Applied Biosystem) were used. The following primers were designed (synthesis by MWG): *LOX2* (lipoxygenase 2: At3g5140): forward 5'-TAC TTG CCT TCC CAA ACA CC-3'; reverse 5'-AGT GCC CTT GGC TGT AGA GA-3'; *ABF2* (*AREB1*) (At1g45249): forward TGG AGG TGG AGG GTT GAC TA-3', reverse 5'-CAT CCT TGT TCA TTG ACC CA-3'; *ABF4* (*AREB2*) (At3g19290): forward GTA GTG TCA TGC CCT TGG CT-3', reverse 5'-ATC GAC CCG AAA TCT TTT CC, *CER5* (At5g1500): forward 5'-GTC CGA CTC

GAA GAT TGC TC-3', reverse 5'-TCG TTG ACT TCT TCT TTG GTC A-3'; *CER6* (At5g43760): 5'-ATC GAC GAG CTC CAA AAG AA-3', reverse 5'-TTA CAT TTC CAC ACG GCA GA-3'; *RD29B*, (At5g52300): forward 5'-GCA CCA CCG TTG GGA CTA TG, reverse 5'-CCA CTG CCT CCA ACT CAC TT-3'; 18SrRNA forward 5'-CGT CCC TGC CCT TTG TAC AC-3', reverse 5'-AAC ACT TCA CCG GAC CAT TCA; MPK3 (At3g45640): forward 5'-GAC AGA GTT GCT TGG CAC AC-3', reverse 5'-CCT CAT CCA GAG GCT GTT GT-3'; *PEPC* (At2g42600): forward 5'-TTG AGG GTA ACG GTT CAA GG-3'; reverse 5'-CAC GGG TAA GTG AAC CTC GT-3'; actin 2 (At3g18780): forward 5'-TGC CAA TCT ACG AGG GTT TC-3', reverse 5'-TTC TCG ATG GAA GAG CTG GT-3'. The cDNA was diluted 10-fold with water and used for real time PCR in a final volume of 10 µl. The PCR conditions consisted of denaturation at 95°C, for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.[63] At the end of each run, a dissociation curve was generated to ascertain single product amplification (Applied Biosystem software, Applied Biosystems fast real time PCR 7500).

All transcript levels were measured with at least four independent biological replicates, each biological replicate was analyzed in three technical replicates. 18s RNA was used to normalize expression data between samples. Negative control reactions without template were routinely performed.

In vivo cytoskeleton visualization. For in vivo actin visualization an *Arabidopsis thaliana* line stably transformed with *35S::GFP:FABD* construct was used as reliable marker for filamentous actin.[64] For in vivo visualization of microtubules an *Arabidopsis thaliana* line stably transformed with *35S::GFP:MBD* construct was used as reliable marker for microtubular cytoskeleton.[65] Seedlings were treated with 10 mg camphor/L and 5 mg menthol/L for 48 h as a maximum (within 24 h, 10 mg menthol evaporate at room temperature). Stomata cells of the treated cotyledons and those of control plants were analyzed for effects of the cytoskeleton by using Olympus FV1000 confocal laser scanning microscopy system equipped with Argon Laser using 488 nm wavelength, and operated with the FV10-ASW1.7 software (Olympus, Hamburg, Germany). We investigated at least 30 stomata complexes in cotyledons of 3 seedlings. This experiment was repeated 3 times. The variability in cytoskeletal arrangements was low and the presented images are the most representative ones.

Plant cultivation and camphor treatment for non-invasive studies. Plants of *Arabidopsis thaliana* ecotype Col-0 were grown under controlled conditions at 22°C/18°C, 170 µmol m^{-2} s^{-1} PAR, and an 8 h/16 h day/night regime. After cotyledon unfolding, single plants were transferred into pots filled with a mixture of potting soil and sand 67 vol.-% potting soil (De Ceuster Meststoffen SA/NV, Grobbendonk, Belgium; 33 vol.-% sand [quartz, grain size 0.7 to 1.4 mm, Rheinische Baustoffwerke, Weilerswist, Germany]). 40 pots (7 cm x 7 cm x 8 cm) were arranged on a tray and were watered thoroughly immediately after pricking out the plants.

For non-invasive studies, plants were fumigated with camphor by placing the pots together with a camphor-containing dish into a 76.8 L plastic box covered with transparent foil. Ten plants were placed in one box. For treatment, the dish contained 768 mg of camphor (i.e., 10 mg for each liter air volume, 39 mg will evaporate within 24 h at room temperature) and for control plants the dish was empty. Fumigation took place for 96 h followed by 72 h without treatment and then a second 96 h-fumigation period.

Data acquisition for non-invasive studies. Repeated measurements of plant size, morphology and chlorophyll fluorescence were carried out with GROWSCREEN-FLUORO. The tray with 40 plant pots was placed under the measuring unit, which moved from one pot to the next and acquired images according to a predefined protocol.[45] Image processing yielded in information on plant size, number of leaves, surface coverage, eccentricity, stockiness and quantum yield for each plant. Plant sizes were acquired as projected leaf area (A_{PT}), which is a good proxy for plant biomass.[65] Relative growth rates (RGR in $\%d^{-1}$) were calculated from subsequently measured A_{PT} values (A_1, A_2) according to $RGR = 100 \times 1/t \times \ln(A_2/A_1)$ with "t" indicating the time between the acquisitions of A_1 and A_2.

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