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Original Article

Volatile compounds emitted by diverse phytopathogenic microorganisms promote plant growth and flowering through cytokinin action

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ABSTRACT

It is known that volatile emissions from some beneficial rhizosphere microorganisms promote plant growth. Here we show that volatile compounds (VCs) emitted by phylogenetically diverse rhizosphere and non-rhizhosphere bacteria and fungi (including plant pathogens and microbes that do not normally interact mutualistically with plants) promote growth and flowering of various plant species, including crops. In *Arabidopsis* plants exposed to VCs emitted by the phytopathogen *Alternaria alternata*, changes included enhancement of photosynthesis and accumulation of high levels of cytokinins (CKs) and sugars. Evidence obtained using transgenic *Arabidopsis* plants with altered CK status show that CKs play essential roles in this phenomenon, because growth and flowering responses to the VCs were reduced in mutants with CK-deficiency (35S:AtCKX1) or low receptor sensitivity (*ahk2/3*). Further, we demonstrate that the plant responses to fungal VCs are light-dependent. Transcriptomic analyses of *Arabidopsis* leaves exposed to *A. alternata* VCs revealed changes in the expression of light- and CK-responsive genes involved in photosynthesis, growth and flowering. Notably, many genes differentially expressed in plants treated with fungal VCs were also differentially expressed in plants exposed to VCs emitted by the plant growth promoting rhizobacterium *Bacillus subtilis* GB03, suggesting that plants react to microbial VCs through highly conserved regulatory mechanisms.

Key-words: cytokinin; flowering; growth promotion; microbial volatile compounds; photoregulation; photosynthesis; plant–microbe interaction; starch.

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INTRODUCTION

Plants' growth and development are influenced by microorganisms occurring either aboveground in the phyllosphere, underground in the rhizosphere and/or in the endosphere inside the vascular transport system and apoplastic space. Microbes synthesize a multitude of substances including carbohydrates, proteins, lipids, amino acids and hormones, which may act directly or indirectly to activate plant immunity or regulate plant growth and morphogenesis (De-la-Peña & Loyola-Vargas 2014). Microbes also synthesize and emit many volatile compounds (VCs) with molecular masses less than 300 Da, low polarity and a high vapor pressure (Schulz & Dickschat 2007; Lemfack *et al.* 2014) that can diffuse far from their point of origin and migrate in soil and aerial environments as well as through porous wood materials. Hence, VCs may play potentially important roles as semiochemicals in interspecies communication, participating in countless interactions among plants and microorganisms, both belowground and aboveground (Kanchiswamy *et al.* 2015).

Mixtures of VCs emitted by some bacteria and fungi can exert inhibitory effects on plant growth (Splivallo *et al.* 2007; Tarkka & Piechulla 2007; Wenke *et al.* 2012; Weise *et al.* 2013). Conversely, depending on microbial culture conditions, volatile emissions from some beneficial rhizosphere bacteria and fungi can promote plant growth (Ryu *et al.* 2003; Blom *et al.* 2011; Hung *et al.* 2013; Meldau *et al.* 2013; Naznin *et al.* 2013; Bailly *et al.* 2014). Although these effects were largely attributed to the two volatiles 3-hydroxybutan-2-one and 2,3-butanediol, several studies have identified additional microbial bioactive VCs that promote plant growth (von Rad *et al.* 2008; Zou *et al.* 2010; Blom *et al.* 2011; Velázquez-Becerra *et al.* 2011; Groenhagen *et al.* 2013; Meldau *et al.* 2013; Naznin *et al.* 2013; Bailly *et al.* 2014). An analysis of *Arabidopsis* mutants with perturbations in hormone production and signalling, in conjunction with analyses of hormone contents, has indicated that abscisic acid (ABA), auxins and cytokinins (CKs) (but not

ethylene, brassinosteroids and gibberellins) may participate in the growth-promoting effect of VCs emitted by the beneficial *Bacillus subtilis* (strain GB03) bacterium, suggesting the involvement of complex signalling mechanisms (Ryu *et al.* 2003; Zhang *et al.* 2007, 2008). Microbial VCs can also promote changes in plants' photosynthetic capacity and transitions from source to sink status in photosynthetic tissues. For example, volatile emissions from *B. subtilis* GB03 augment photosynthetic capacity by increasing photosynthetic efficiency and chlorophyll content in *Arabidopsis* (Zhang *et al.* 2008). Furthermore, VCs from a number of microorganisms ranging from Gram-negative and Gram-positive bacteria to different fungi promote accumulation of exceptionally high levels of starch in leaves of mono-cotyledonous and di-cotyledonous plants (Ezquer *et al.* 2010; Li *et al.* 2011).

To date, studies on stimulatory effects of microbial VCs on plant growth have mainly focused on a few beneficial rhizosphere bacteria and fungi, using *Arabidopsis* plants cultured in Murashige and Skoog (MS) medium supplemented with sucrose as model systems (Ryu *et al.* 2003; Zhang *et al.* 2007, 2008; von Rad *et al.* 2008; Kwon *et al.* 2010; Zou *et al.* 2010; Groenhagen *et al.* 2013; Hung *et al.* 2013). Exogenously added sucrose inhibits expression of photosynthetic genes (Jang & Sheen 1994; Osuna *et al.* 2007) and may trigger senescence and growth arrest in plants (Ohto *et al.* 2001; Teng *et al.* 2005). To increase knowledge of the extent and nature of microbial VCs-mediated interactions between plants and microorganisms in this work we assessed responses of *Arabidopsis* and other plants cultured on sucrose-free medium to VCs emitted by phylogenetically diverse rhizosphere and non-rhizosphere bacteria and fungi, including some pathogenic strains. We found that all the tested microorganisms produced VCs that promoted growth and flowering, suggesting that this action is not restricted to some beneficial rhizosphere bacteria and fungi but extends to pathogens and microbes that are not normally considered to interact mutualistically with plants. Thus, to obtain insights into the mechanisms involved in the microbial VCs-mediated promotion of growth and flowering we also characterized *Arabidopsis* plants exposed to the VCs emitted by the opportunistic fungal plant pathogen *Alternaria alternata*. We found that promotion of growth and flowering by VCs emitted by this fungus involves a highly conserved and complex network of transcriptionally regulated processes allowing the plant to acclimate to the new environmental conditions imposed by the VCs treatment wherein light and CK signalling play an important role. The discovery that VCs from pathogenic microorganisms can have beneficial effects on plant growth and development extends knowledge of the diversity and complexity of the interactions involved in modulation of plant physiology, raising questions regarding the evolution of the processes, their ecological significance and potential applications.

MATERIALS AND METHODS

Plant and microbial cultures and growth conditions

The work was carried out using *A. thaliana* (Heynh) (ecotypes Col-O and Ws-2) and CK deficient, CK oxidase/dehydrogenase 1 over-expressing 35S:CKX1 plants (Werner *et al.* 2003) and CK signalling *ahk2/3*, *ahk2/4* and *ahk3/4* mutants (Riefler *et al.* 2006). We also used maize (*Zea mays*, cv. HiII) and pepper (*Capsicum annuum*, cv. Sweet Italian) plants. Microorganisms used in this study are listed in Supporting Information Table S1. Unless otherwise indicated *Arabidopsis* plants were cultured in Petri dishes containing sucrose-free solid MS (Duchefa Biochemie M0222) medium in growth chambers with a 16 h light (90 μmol photons s^{-1} m^{-2})/8 h dark photoperiod (22 °C during the light period and 18 °C during the dark period). Bacteria were cultured in Petri dishes containing solid M9 minimal (95 mM Na_2HPO_4/44 mM KH_2PO_4/17 mM NaCl/37 mM NH_4Cl/0.1 mM $CaCl_2$/2 mM $MgSO_4$, 1.5% bacteriological agar) medium supplemented with 50 mM glucose. M9 medium for *B. subtilis* culture was supplemented with 7 μM each of $MnSO_4$, $FeSO_4$ and $ZnSO_4$, and 1 μM thiamine. Fungi were cultured in Petri dishes containing solid MS medium supplemented with 90 mM sucrose. To investigate effects of microbial VCs on *Arabidopsis* plants cultured in MS medium, microbial and plant cultures without lids were placed without physical contact into sterile plastic boxes (IT200N Instrument Try 200 × 150 × 50 mm, AWGregory, UK) and sealed with a plastic film as illustrated in Supporting Information Fig. S1a. Effects of microbial VCs on plants cultured on soil were investigated by placing microbial cultures without lids and plants in sealed mini-green houses as illustrated in Supporting Information Fig. S1b,c. As negative control, plants were cultured together with adjacent Petri dishes containing sterile microbial culture media. Unless otherwise indicated microbial VCs treatment started at the 14 days after sowing (DAS) growth stage of plants.

Gas exchange determinations

Changes in photosynthetic capacity and mitochondrial respiration of leaves upon exposure to microbial VCs were investigated essentially as described by Bahaji *et al.* (2015b). Briefly, fully expanded apical leaves were enclosed in a LI-COR 6400 gas exchange portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA). The gas exchange determinations were conducted at 25 °C with a photosynthetic photon flux density of 350 μmol m^{-2} s^{-1}. Net rates of CO_2 assimilation (A_n) were calculated using equations developed by von Caemmerer & Farquhar (1981). From the A_n/*Ci* curves, the maximum rate of carboxylation by Rubisco (V_{cmax}), triose phosphate use (TPU) and the maximum electron transport demand for RuBP regeneration (J_{max}) values were calculated according to Long & Bernacchi (2003). To avoid miscalculation of A_n and intercellular CO_2 concentration (*Ci*) because of leakage into the gasket of the gas analyser, we performed CO_2 response curves using an empty chamber. The values obtained for A_n and *Ci* in the empty chamber were compared with those of the chamber filled with a leaf and subtracted from the values obtained with the empty chamber. The photosynthetic electron transport rate (ETR) values were calculated according to Krall & Edwards (1992) as photosystem II (PSII) operating efficiency (Φ_{PSII}) × PPFD × 0.84 × 0.5, where PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 was used as the fraction of excitation energy distributed

to PSII (Ögren & Evans 1993) and 0.84 as the fractional light absorbance (Morales *et al.* 1991). The rate of mitochondrial respiration in the dark was determined by measuring the rate of CO_2 evolution in the dark.

Chlorophyll fluorescence emission parameters were determined using a PlantScreen^{TM} XYZ System (Photon Systems Instruments, Brno, Czech Republic). This phenotyping system is equipped with a FluorCam unit for pulse amplitude modulated measurement of chlorophyll fluorescence. After 20 min of dark adaptation the standardized measuring protocol was applied, as described in Humplík *et al.* (2015). The maximum quantum yields of PSII in the dark-adapted state (Φ_{Po}) (also referred to as F_v/F_m), Φ_{PSII} and non-photochemical quenching (Φ_{NPQ}) were calculated from the measured parameters according to Lazár (2015).

Analytical methods

Fully expanded source leaves of plants cultured in the absence or presence for 3 days of VCs were harvested at the end of the light period, freeze-clamped and ground to a fine powder in liquid nitrogen with a pestle and mortar. For measurement of sucrose, glucose and fructose, a 0.1 g aliquot of the frozen powder was resuspended in 1 mL of 90% ethanol, left at 70 °C for 90 min and centrifuged at 13 000 *g* for 10 min. Sugar contents from supernatants were then determined by HPLC with pulsed amperometric detection on a DX-500 Dionex system as described in Bahaji *et al.* (2015a). Glyceraldehyde 3-P (GAP) and 3-phosphoglycerate (3PGA) contents were determined as described by Vogt *et al.* (1998) and Lytovchenko *et al.* (2002), respectively. Starch was measured by using an amyloglucosydase-based test kit (Boehringer Mannheim, Germany). Total carotenoid and chlorophyll contents were quantified according to Lichtenthaler (1987). To determine CK levels, portions of the frozen leaves (refer to the preceding texts) from Ws-2 plants were lyophilized and CKs were quantified according to the method described in Novák *et al.* (2008). ABA content was determined essentially as described by Floková *et al.* (2014).

Gene expression analyses

Total RNA was extracted from frozen *Arabidopsis* leaves of *in vitro* cultured plants using the Trizol method according to the manufacturer's procedure (Invitrogen), following purification with RNeasy kit (Qiagen). RNA amplification, labelling and statistical data analysis were performed basically as described by Adie *et al.* (2007). The *Arabidopsis* Gene Expression Microarray 4 × 44 K (G2519, Agilent Technologies) was used for hybridization. Labelling and hybridization conditions were those described in 'The manual two colour microarray based gene expression analysis' of Agilent Technologies. Three independent biological replicates were hybridized for leaves from microbe-treated plants and from controls. Images from Cy3 and Hyper5 channels were equilibrated for intensity differences and captured with a GenePix 4000B scanner (Axon). Spots were quantified using GenPix software (Axon) and normalized using the Lowess method. Means of the three replicate log-ratio intensities and their standard deviations were calculated, and the expression data were statistically analysed using the LIMMA Package (Smyth & Speed 2003). Functional characterization of the differentially expressed genes was performed using the MapMan tool (http://gabi.rzpd.de/projects/MapMan/).

Real-time quantitative PCR

Total RNA was extracted from *Arabidopsis* leaves as described earlier for the microarray analyses, then treated with RNAase free DNAase (Takara). RNA (1.5 μg) was reverse transcribed using polyT primers and an Expand Reverse Transcriptase kit (Roche) according to the manufacturer's instructions. RT-PCR reaction was performed using a 7900HT sequence detector system (Applied Biosystems) with the Premix Ex Tag Mix (Takara RR420A) according to the manufacture's protocol. Each reaction was performed in triplicate with 0.4 μl of the first strand cDNA in a total volume of 20 μl. The specificity of the PCR amplifications was checked by acquiring heat dissociation curves (from 60 to 95 °C). Comparative threshold values were normalized to 18S RNA internal control and compared with obtain relative expression levels. Primers used for RT-PCRs are listed in Supporting Information Table S2, and their specificity was checked by separating the obtained products on 1.8% agarose gels.

Statistical analysis

Presented data are the means (±SE) of four independent experiments, with 3–5 replicates for each experiment. The significance of differences between VC-treated and non-treated plants was statistically evaluated with Student's *t*-test using the SPSS software. Differences were considered significant if $P < 0.05$. In hormone content analyses, significance was determined by ANOVA for parametric data and Kruskal–Wallis for non-parametric data, using the open source R software 2.15.1 (http://cran.r-project.org/). Multiple comparisons after ANOVA were calculated using the post hoc Tukey's honestly significant difference test.

RESULTS

Volatile compounds emitted by phylogenetically diverse microorganisms other than beneficial rhizosphere bacteria and fungi promote plant growth and flowering

Arabidopsis plants were cultured on sucrose-free solid MS medium in the absence or continuous presence of adjacent cultures of phylogenetically diverse strains of beneficial and non-beneficial fungi and bacteria. These experiments were conducted in sterile growth boxes with no physical contact between the plant and the microbial cultures (Supporting Information Fig. S1a). VCs emitted by all the tested microorganisms (including plant pathogens) induced twofold to fivefold increases in

fresh weight (FW) of the *Arabidopsis* plants, relative to controls (Fig. 1a). VCs from most of microorganisms also induced early flowering (Fig. 1b,c). Consistent with our previous studies (Ezquer *et al.* 2010), VCs also promoted the accumulation of exceptionally high levels of starch (Supporting Information Fig. S2). The strength of the responses to microbial VCs differed from one microorganism to another (Fig. 1; Supporting Information Fig. S2), which can be ascribed to activation of different signalling pathways in plants in response to different mixtures of VCs emitted by the microorganisms.

To assess further the generality of these responses we grew *Arabidopsis* and two plant species of agronomic interest, sweet pepper and maize, on soil (Supporting Information Fig. S1b,c) and examined their responses to microbial VCs. The microbial VC-exposed *Arabidopsis* plants had significantly higher FW than controls within 4 days of the treatment and twice as high FW after another 7 days (Fig. 2a). In addition, exposed maize and pepper plants were almost twice as tall as controls from day 22 of the treatment until the end of experiment on day 47 (Fig. 2b,c).

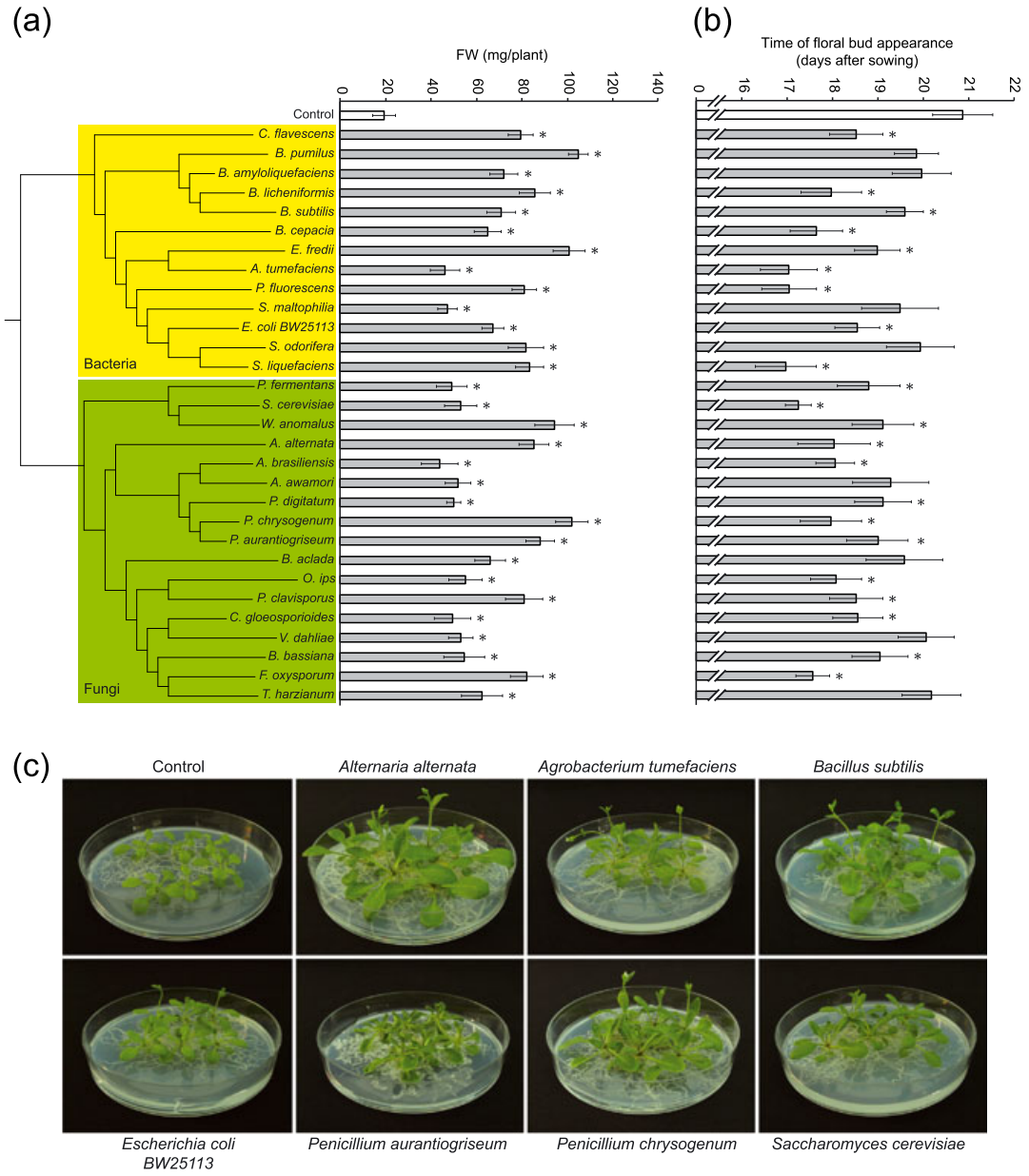

Figure 1. VCs emitted by phylogenetically diverse microorganisms promote plant growth and flowering. (a) Rosette FW, (b) time of floral bud appearance and (c) external phenotypes of *Arabidopsis* plants cultured in the absence or continuous presence of adjacent cultures of the indicated microorganisms for one week. In 'a' and 'b' values represent the means ± SE determined from four independent experiments using 12 plants in each experiment. Asterisks indicate significant differences between microbial VC-treated plants and controls (non-treated plants) according to Student's *t*-tests ($P < 0.05$). The phylogenetic tree was constructed using the PhyloT phylogenetic tree generator (www.phyloT.biobyte.de).

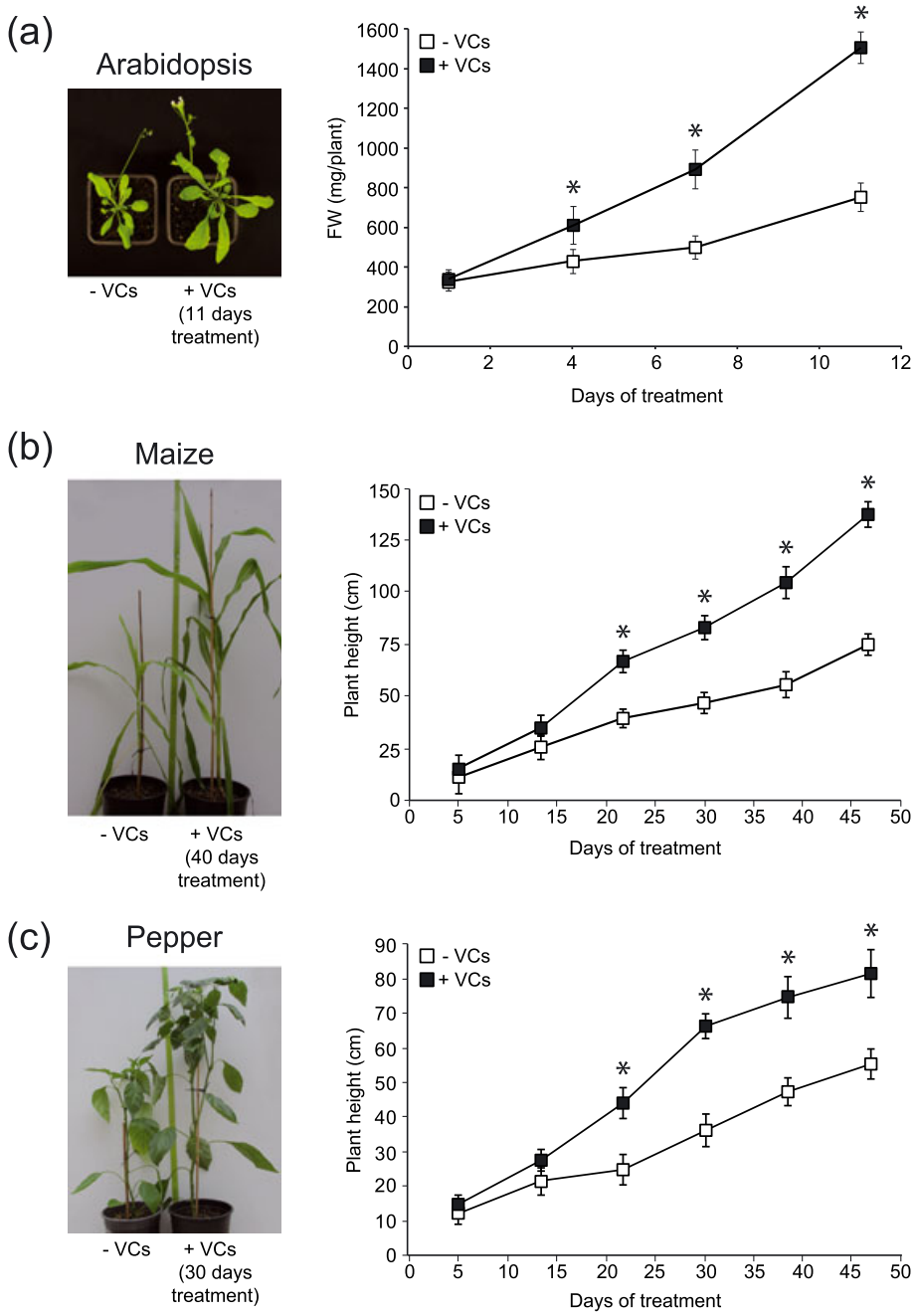

Figure 2. Microbial VCs promote growth of soil-grown *Arabidopsis*, maize and pepper plants. (a) Rosette FW of *Arabidopsis* plants cultured on soil in the absence or continuous presence of adjacent cultures of *A. alternata* for indicated times. Essentially, the same results were obtained using cultures from other bacterial and fungal species (not shown). Height of soil-grown maize (b) and pepper (c) plants cultured in the absence or continuous presence of adjacent cultures of *A. alternata* for indicated times. Values represent the means ± SE determined from four independent experiments using 12 plants in each experiment. Asterisks indicate significant differences between VC-treated and non-treated plants according to Student's *t*-tests ($P < 0.05$).

Alternaria alternata volatile compounds increase photosynthetic activities of exposed plants

We measured key parameters of light and dark phases of photosynthesis in plants exposed to *A. alternata* VCs for 3 days. During the light phase Φ_{Po} and Φ_{PSII} were higher, and Φ_{NPQ} significantly weaker, in leaves of the exposed plants than in controls (Table 1). These results indicate that leaves of VC-treated plants used the light more efficiently, dissipated less excitation energy as heat, reduced more $NADP^+$, formed more ATP and hence had higher A_n than controls. This inference was corroborated by the analysis

Table 1. Photosynthetic parameters of leaves of plants cultured in the absence or presence of VCs emitted by *A. alternata* for 3 days

Treatment	Φ_{Po}	Φ_{PSII}	Φ_{NPQ}	J_{max} (μmol e^- m^{-2} s^{-1})	V_{cmax} (μmol CO_2 m^{-2} s^{-1})	TPU (μmol Pi m^{-2} s^{-1})
−VCs	0.70 ± 0.01	0.33 ± 0.02	0.81 ± 0.04	45.54 ± 1.13	18.81 ± 0.47	2.58 ± 0.08
+VCs	0.83 ± 0.01	0.44 ± 0.01	0.68 ± 0.03	56.61 ± 2.89	29.52 ± 0.91	3.16 ± 0.09

Values are means ± SE from four independent experiments.

of the levels of photosynthetic pigments and A_n under varying *Ci*. As shown in Fig. 3a, total chlorophyll and carotenoid contents in leaves of VC-treated *pgi1-2* plants were higher than in controls. Moreover, plants exposed to VCs had higher A_n than controls at all *Ci* levels (Fig. 3b). V_{cmax} and J_{max} determined from the A_n/*Ci* curves were significantly higher in leaves of VC-treated plants than in controls, as was TPU (Table 1). Furthermore, ETR was higher in the VC-treated plants than in controls (Fig. 3c), particularly under minimal *Ci* conditions (in which the ETR was fourfold higher than in controls). Levels of soluble carbohydrates, regarded here as primary photosynthates, were significantly up-regulated by *A. alternata* VCs. As shown in Fig. 4, the contents of sucrose, glucose, fructose and Calvin–Benson cycle intermediates such as GAP and 3PGA were higher in leaves of VC-treated plants than in controls.

Alternaria alternata volatile compounds augment the levels of active forms of plastidic CKs

ABA and CKs are important determinants of photosynthesis. To investigate the possible involvement of these hormones in the responses to *A. alternata* VCs we measured their levels in mature leaves of plants cultured in the absence or presence of adjacent cultures of *A. alternata* for 3 days. *Alternaria alternata* VCs promoted a moderate, statistically non-significant reduction of the ABA content in leaves (224.48 ± 35.31 and 299.54 ± 37.27 pmol g^{-1} DW in leaves of VC-treated and non-treated plants, respectively). In clear contrast, fungal VCs caused a significant increase of the total content of plastidic-type, 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway-derived CKs (Table 2; Supporting Information Fig. S3). The most strongly accumulated CK forms were the ribosides of isopentenyladenine (iP) and *trans*-zeatin (tZ) (iPR and tZR, respectively) and their precursors (iPRMP and tZRMP, respectively), levels of which increased threefold. Levels of free bases of the most biologically active iP and tZ increased 1.5-fold (Table 2; Supporting Information Fig. S3), whereas levels of the less biologically active CKs dihydroxy zeatin (DZ) and *cis*-zeatin (cZ) were substantially reduced (3- and 2-fold, respectively). Concentrations of inactive *N*- and *O*-glycosylated forms were not significantly affected by *A. alternata* VC exposure, except that iP7G and DZ7G levels were slightly lower in the treated plants. The pool of glycosylated forms of cZ was 1.5-fold lower, mainly because of reductions in cZ9G and cZOG concentrations (Table 2).

CKs and CK signalling are required for activities of *Alternaria alternata* VCs

We compared responses to VCs between wild-type (WT) *Arabidopsis* plants, CK-deficient 35S:CKX1 transgenic plants (Werner *et al.* 2003) and double CK receptor knock-out mutants with impaired sensitivity to CKs (*ahk2/3*, *ahk2/4* and *ahk3/4*) (Riefler *et al.* 2006). As shown in Fig. 5a, VC-promoted increase of rosette FW in *ahk2/4* and *ahk3/4* plants was comparable to that of WT plants, implying that, individually or in combination with AHK2 or AHK3, AHK4 plays a minor role in VCs signalling and subsequent growth promotion. The magnitude of this phenomenon in 35S:CKX1 and *ahk2/3* plants was markedly reduced when compared with WT plants (Fig. 5a). Similar to WT plants, the appearance of floral buds in VCs treated *ahk2/4* and *ahk3/4* plants occurred 3–4 days before non-treated plants (Fig. 5b). In clear contrast, VCs did not exert any significant effect on the time of floral bud appearance in both *ahk2/3* and 35S:CKX1 plants (Fig. 5b). VCs promoted the accumulation of exceedingly high levels of starch in leaves of *ahk2/4* and *ahk3/4* plants, but their effect was markedly reduced in 35S:CKX1 and *ahk2/3* plants (Fig. 5c). These findings provide strong evidence that *A. alternata* VC-promoted enhancement of aerial growth, early floral bud appearance and starch accumulation is strongly regulated by CKs and indicate that these responses are mediated mainly through AHK2 and AHK3 receptors.

Plant responses to *Alternaria alternata* volatile compounds are light-dependent and subject to photoperiod control

CKs serve as endogenous cues that strongly influence plants' responsiveness to light (Guo *et al.* 2005; Kieber & Schaller 2013; Cortleven & Schmülling 2015), suggesting that some of the processes promoted by *A. alternata* VCs might be, at least partially, photoregulated. To test this hypothesis, we compared the rosette FW, flowering and leaf starch contents of plants cultured under a 16 h light/8 h dark photoperiod that were exposed to *A. alternata* VCs for 1 week either only during the light phases or only during the dark phases. Exposure to VCs only during the light phases promoted growth (Fig. 6a), starch over-accumulation (Fig. 6b) and flowering (Fig. 6c). In clear contrast, exposure to VCs only during the dark phases had no effect on the plants' external phenotype (Fig. 6a,c) and did not induce starch over-accumulation in their leaves (Fig. 6b). These findings strongly indicate that *A. alternata* VC-promoted changes in the treated plants are light-dependent.

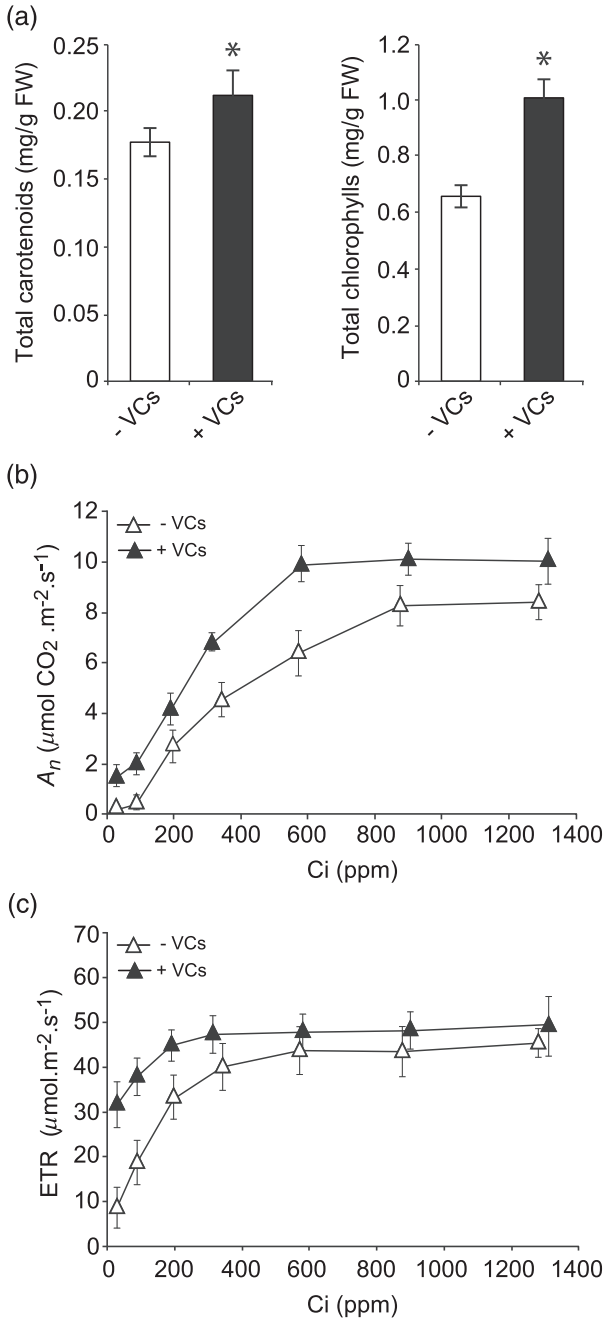

Figure 3. *A. alternata* VCs enhance photosynthesis in exposed plants. (a) Total chlorophyll and carotenoids contents, curves of (b) net CO_2 assimilation rate (A_n), and (c) photosynthetic electron transport (ETR) versus intercellular CO_2 concentration (*Ci*) in leaves of plants cultured in the absence or continuous presence of adjacent cultures of *A. alternata* for 3 days. VCs treatment started at the 18 DAS growth stage of plants. In a values represent the means ± SE determined from four independent experiments using 12 plants in each experiment. Asterisks indicate significant differences between leaves of VC-treated and control (non-treated) plants according to Student's *t*-tests ($P < 0.05$).

Volatile compounds emitted by *Alternaria alternata* and the plant growth promoting rhizobacterium *Bacillus subtilis* induce similar transcriptomic changes in *Arabidopsis* leaves

The next step in the study presented here was a high-throughput transcriptome analysis of leaves from *Arabidopsis* plants cultured *in vitro* under a 16 h light/8 h dark photoperiod in the absence or in the presence for 16 h of VCs emitted by *A. alternata*. As shown in Supporting Information Table S3, this analysis using an *Arabidopsis* Gene Expression Microarray 4 × 44 K (G2519, Agilent Technologies) revealed that 530 genes were up-regulated and 496 genes were down-regulated in the presence of VCs (with a ≥2.0-fold difference relative to control; $P < 0.05$). Quantitative real-time RT-PCR analyses of some of the identified genes (Supporting Information Fig. S4) validated the results of the array analyses. To determine the biological processes affected by VCs, an analysis of genes using the MapMan tool (Thimm *et al.* 2004) (http://gabi.rzpd.de/projects/MapMan/) was carried out. This study revealed that *A. alternata* VCs promote changes in the expression of *Arabidopsis* genes involved in multiple processes including light harvesting, starch synthesis and breakdown, flowering, cell wall biosynthesis, anthocyanin and carotenoid metabolism and protection against oxidative stress. (Fig. 7). Notably, ~7% of the differentially regulated genes are known to be CK responsive (Supporting Information Table S3). Furthermore, a significant number of the VC-responding genes are known to be regulated by light, auxin, ethylene, jasmonic acid, gibberellin, nitrate and sugars, indicating intense crosstalk between environmental cues, hormones and metabolites.

VCs emitted by the plant growth promoting rhizobacterium (PGPR) *B. subtilis* GB03 promote drastic changes in the transcriptome of *Arabidopsis* (Zhang *et al.* 2007). As the volatilomes of bacteria and fungi are very different (Schulz & Dickschat 2007; Lemfack *et al.* 2014) we hypothesized that changes in the transcriptome of plants triggered by mixtures of VCs emitted by *B. subtilis* GB03 could differ from those triggered by *A. alternata* VCs. Thus, we compared the sets of genes differentially expressed in leaves of plants exposed to *A. alternata* VCs (Supporting Information Table S3) with those of leaves of plants exposed to VCs emitted by *B. subtilis* GB03 (cf. Supporting Information Table S1 in Zhang *et al.* 2007). Contrary to our expectations, we found that 101 out of 254 genes that are down-regulated in leaves of plants exposed to VCs emitted by *B. subtilis* GB03 (including 85% of the 20 most strongly down-regulated genes) are also down-regulated in leaves of plants exposed to VCs emitted by *A. alternata* (Table 3; Supporting Information Table S4). Furthermore, 99 out of the 378 genes that are up-regulated in leaves of plants exposed to VCs emitted by *B. subtilis* GB03 (including 70% of the 20 most strongly up-regulated genes) are also up-regulated in leaves of plants exposed to VCs emitted by *A. alternata* (Table 3; Supporting Information Table S5). Notably, ~25% of the genes that are differentially regulated in *Arabidopsis* leaves exposed to VCs emitted by both *B. subtilis* and *A. alternata* are CK responsive genes (Table 3).

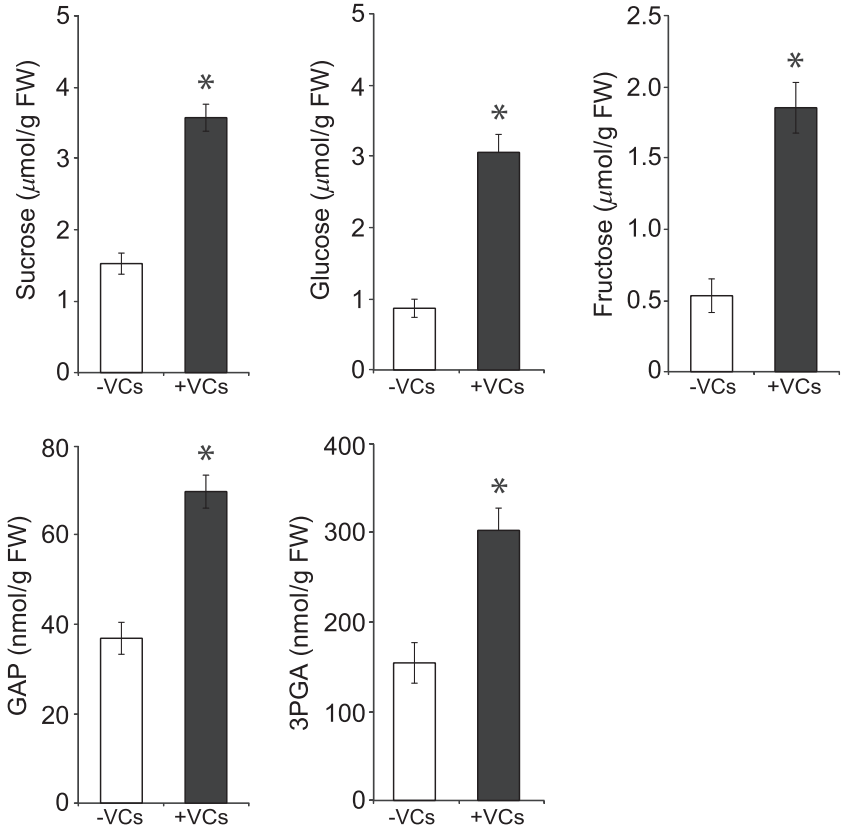

Figure 4. *A. alternata* VCs increase soluble sugar levels in leaves. Soluble carbohydrate contents were estimated in leaves of plants grown in the absence or continuous presence of adjacent cultures of *A. alternata* for 3 days. Leaves were harvested at the end of the light period. Values represent the means ± SE determined from four independent experiments using 12 plants in each experiment. Asterisks indicate significant differences between leaves of VC-treated and control (non-treated) plants according to Student's *t*-tests ($P < 0.05$).

DISCUSSION

Many plant pathogenic bacteria and fungi have evolved to interact with plants, exhibiting a versatile metabolism and ingenious mechanisms tailored to modify the development of their hosts. Consequently, it has been suggested that phytopathogens or their constituents may provide opportunities for plant production or be useful for specific biotechnological applications (Tarkowski & Vereecke 2014). In line with this opinion, in this work we have shown that blends of VCs emitted by a number of beneficial and non-beneficial, phylogenetically diverse microorganisms (including plant pathogens) promote growth and flowering in both mono-cotyledonous and di-cotyledonous plants (Figs 1 and 2). As to the ecological implication of this phenomenon we can just speculate that plant growth promotion by microbial VCs prepares the plant to host the microorganism, which in the case of phytopathogenic microorganisms ensures proper continuation into the pathogenic phase.

Bioprospecting of VCs from non-beneficial microorganisms and characterization of their biological functions and ecological roles could offer valuable new strategies for increasing yield of horticultural crops or biotechnological products in a sustainable and environmentally benign manner. We must emphasize that part of our future goal is to identify microbial VCs promoting plant growth. The fact that mixtures of VCs emitted by all microbial species analysed in this work promote growth would indicate that plants respond to a wide range of bioactive VCs, as strongly supported by previous reports using pure VCs emitted by different microbial species (Ryu *et al.* 2003; Zou *et al.* 2010; Blom *et al.* 2011; Velázquez-Becerra *et al.* 2011; Groenhagen *et al.* 2013; Meldau *et al.* 2013; Naznin *et al.* 2013). Alternatively and/or additionally, it is likely that all microorganisms emit the same VCs promoting plant growth. In this respect, it is worth to note that all microbial species tested in this work produce CO_2. Although this would indicate in principle that VC-treated plants were exposed to elevated CO_2 (a situation that would favour growth because of enhanced photosynthetic CO_2 fixation) we failed to detect substantial increases of CO_2 levels in the growth boxes upon inclusion of cultures of most of microbial species used in this work (not shown), strongly indicating that the positive effect exerted by microbial VCs on plant growth is not ascribed to photosynthetic fixation of CO_2 emitted by the microorganisms.

Volatile compounds emitted by the fungal phytopathogen *Alternaria alternata* enhance photosynthesis in *Arabidopsis*

Physical contact with pathogens very often leads to a decrease in photosynthesis in plants (Berger *et al.* 2007). However,

Table 2. CK content (pmol g^{-1} DW) in leaves of 18 DAS plants cultured in solid MS medium in the absence or presence of VCs emitted by *A. alternata* for 3 days

	MEP pathway (plastid)-derived CKs			MVA pathway (cytosol)-derived CKs		
		−VCs	+VCs		−VCs	+VCs
Precursors	iPRMP	152.03 ± 16.41	460.71 ± 23.28***			
	tZRMP	104.14 ± 3.43	353.00 ± 25.15***	cZRMP	99.69 ± 15.17	139.67 ± 9.05*
	DZRMP	0.80 ± 0.08	1.84 ± 0.18**			
	∑ (%)	256.98 ± 14.07	815.54 ± 14.12***		99.69 ± 15.17	139.67 ± 9.05*
Transport forms	iPR	16.06 ± 1.06	25.86 ± 2.64**			
	tZR	14.84 ± 2.37	47.77 ± 10.43*	cZR	3.90 ± 0.52	6.55 ± 1.43*
	DZR	0.17 ± 0.03	0.32 ± 0.03**			
	∑ (%)	31.07 ± 2.44	73.95 ± 8.78**		3.90 ± 0.52	6.55 ± 1.43*
Active forms	iP	6.71 ± 0.82	8.44 ± 0.54*			
	tZ	8.71 ± 1.63	11.81 ± 0.92*	cZ	2.96 ± 0.25	1.49 ± 0.05**
	DZ	0.09 ± 0.02	0.03 ± 0.01*			
	∑ (%)	16.51 ± 1.74	20.28 ± 0.32*		2.96 ± 0.25	1.49 ± 0.05**
Glycosylated (inactive) forms	iP7G	137.82 ± 6.92	112.7 ± 6.84*			
	tZ7G	154.48 ± 4.24	155.89 ± 3.64			
	DZ7G	31.76 ± 0.90	23.28 ± 0.19***			
	iP9G	23.51 ± 2.40	19.04 ± 1.15	cZ9G	5.30 ± 0.42	2.78 ± 0.11**
	tZ9G	232.69 ± 15.94	217.87 ± 18.40			
	DZ9G	1.82 ± 0.15	1.09 ± 0.40			
	tZOG	58.10 ± 7.61	53.82 ± 3.84	cZOG	15.72 ± 2.10	9.10 ± 0.84*
	DZOG	4.28 ± 0.43	3.42 ± 0.48			
	tZROG	34.59 ± 7.91	33.17 ± 8.32	cZROG	44.11 ± 3.42	33.82 ± 6.27
	DZROG	5.09 ± 0.46	4.71 ± 1.02			
	∑ (%)	684.14 ± 12.80	625.01 ± 18.52		65.13 ± 3.44	45.71 ± 5.10*
TOTAL	∑ (%)	988.69 ± 11.10	1534.78 ± 23.61***		171.68 ± 13.96	193.41 ± 11.86

Levels of CK precursors, transport forms, active forms and glycosylated inactive forms originating from the MEP and mevalonate (MVA) pathways are separately shown. Total sums and corresponding percentage are shown for individual forms. Asterisks indicate significant differences according to ANOVA.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

surprisingly, in this work we found that VCs emitted by the pathogen *A. alternata* have positive effects on photosynthesis in *Arabidopsis* plants (Fig. 3; Table 1), which can be ascribed, at least partially, to very efficient use of light as a consequence of enhanced accumulation of photosynthetic pigments and improved ETR (Fig. 3). Photosynthesis is generally subject to feedback inhibition by elevated sugar levels through a hexokinase-dependent mechanism of glucose sensing that requires ABA signalling (Moore *et al.* 2003; Rolland *et al.* 2006), although this regulatory mechanism does not apply ubiquitously to all cell types under all growth and developmental conditions (Granot *et al.* 2013). Notably, *A. alternata* VC-promoted enhancement of photosynthesis was accompanied by accumulation of high levels of soluble sugars (Fig. 4). However, unlike *B. subtilis* VCs promoting the reduction of ABA levels as a mechanism to suppress sugar sensing inhibition of photosynthesis in *Arabidopsis* (Zhang *et al.* 2008), *A. alternata* VCs treatment resulted in a moderate, statistically non-significant reduction of ABA levels. These findings would indicate that VC-promoted suppression of sugar sensing inhibition of photosynthesis involves mechanism(s) additional and/or alternative to those implicating ABA. As *A. alternata* VCs promote accumulation of CKs (Table 2), and CKs and sugars work antagonistically in gene-regulated responses (Kushwah & Laxmi 2014) it is conceivable that the lack of photosynthetic inhibition by high sugar content in leaves of VC-exposed plants is due, at least partly, to enhanced CK production.

Plant responses to volatile compounds of the fungal phytopathogen *Alternaria alternata* involve enhanced CK production

CKs are major determinants of growth, energy status and photosynthesis in mature leaves (Cortleven & Valcke 2012; Kieber & Schaller 2013; Bahaji *et al.* 2015b). Furthermore, these versatile hormones play important roles in flowering (Nishimura *et al.* 2004; Riefler *et al.* 2006; D'Aloia *et al.* 2011), modulation of sugar-induced anthocyanin accumulation (Guo *et al.* 2005; Das *et al.* 2012) and interaction of the plant with both biotic and abiotic factors (Argueso *et al.* 2012). Moreover, CKs promote starch accumulation in leaves (Werner *et al.* 2008) most likely by regulating the expression of starch metabolism-related genes (Miyazawa *et al.* 1999) and/or enhancing photosynthetic CO_2 fixation. Results presented in Table 2 showing that levels of plastidic MEP-derived CKs in leaves of plants

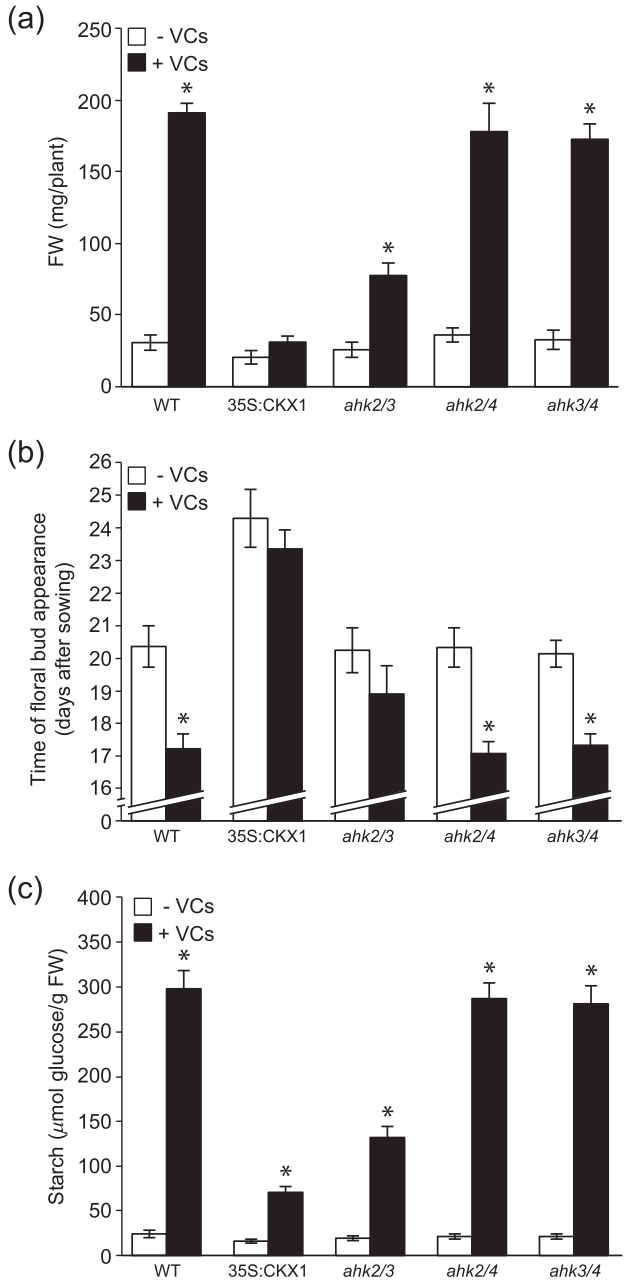

Figure 5. CK signalling is required for activities of *A. alternata* VCs. (a) Rosette FW, (b) time of floral bud appearance, and (c) leaf starch content in WT, 35S:CKX1, *ahk2/3*, *ahk2/4* and *ahk3/4* plants cultured in the absence or continuous presence of adjacent cultures of *A. alternata* for 12 days. Values represent the means ± SE determined from four independent experiments using 12 plants in each experiment. Asterisks indicate significant differences between VC-treated and non-treated plants based on Student's *t*-tests ($P < 0.05$).

treated with *A. alternata* VCs are higher than in non-treated leaves would indicate that enhancement of these CKs is involved in the VC-promoted changes described in this work. This hypothesis is corroborated by the poor responses to VCs observed in 35S:CKX1 and *ahk2/3* plants (Fig. 5).

Regarding mechanisms that may contribute to the high contents of active and transport forms of MEP-derived CKs and their precursors in leaves of *A. alternata* VC-treated plants, it should be noted that the levels of some inactive glycosylated CKs were lower in VC-treated plants than in controls (Table 2; Supporting Information Fig. S3). This would indicate that down-regulation of enzymes involved in the degradation of plastidic CKs could participate in the VC-promoted accumulation of active and transport forms of MEP-derived CKs and their precursors. No significant changes in the expression of genes encoding CK metabolism enzymes could be observed in leaves of *A. alternata* VC-treated plants (Supporting Information Table S3), strongly indicating that VC-promoted enhancement of CKs is largely regulated at the post-transcriptional level. In this respect it should be noted that the first suggested level of diurnal MEP regulation is related to the Calvin–Benson cycle intermediate GAP (Pulido *et al.* 2012; Pokhilko *et al.* 2015). GAP concentrations in chloroplasts fluctuate between 20 μM during the day and 1 μM at night (Arrivault *et al.* 2009). These concentrations are substantially below the Km for GAP (110 μM) of the first enzyme of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (Ghirardo *et al.* 2014), resulting in a strong direct dependence of the MEP pathway flux on the GAP concentration. In VC-treated leaves, GAP concentration is twofold higher than that of non-treated leaves, likely as a consequence of enhanced photosynthesis. Thus, as illustrated in Fig. 8 and Supporting Information Fig. S3, accumulation of high levels of active MEP derived CKs in leaves of VC-treated plants might be at least partly because of enhanced photosynthetic production of GAP and subsequent conversion into MEP-derived CKs. A striking alteration in the transcriptome of *A. alternata* VC-treated plants involves strong up-regulation of *GPT2* (Supporting Information Table S3; Fig. 7), a CK-induced gene (Bhargava *et al.* 2013) encoding a plastidic glucose-6-P (G6P)/Pi transporter, which is necessary for dynamic photosynthetic and metabolic acclimation to increased irradiance (Athanasiou *et al.* 2010; Dyson *et al.* 2015). Therefore, GPT2-mediated incorporation of cytosolic G6P into the chloroplast and subsequent metabolic conversion into GAP linked to the synthesis of CKs (which in turn further promotes *GPT2* expression) may also contribute to the high levels of MEP-derived CKs observed in leaves of VC-treated plants (Fig. 8).

Volatile compounds induce changes in expression of cytokinin- and light-regulated genes involved in photosynthesis, growth, flowering and starch metabolism

Taken together, data presented in this work strongly indicate that changes in VC-exposed plants result from complex, transcriptionally regulated processes allowing the plant to acclimate to new environmental conditions, in which light and CKs play important roles (Fig. 8). *Inter alia*, VCs treatment strongly promoted the expression of a number of light-inducible genes encoding light-harvesting proteins, some of which (e.g. *ELIP1*) are up-regulated by CKs (Supporting

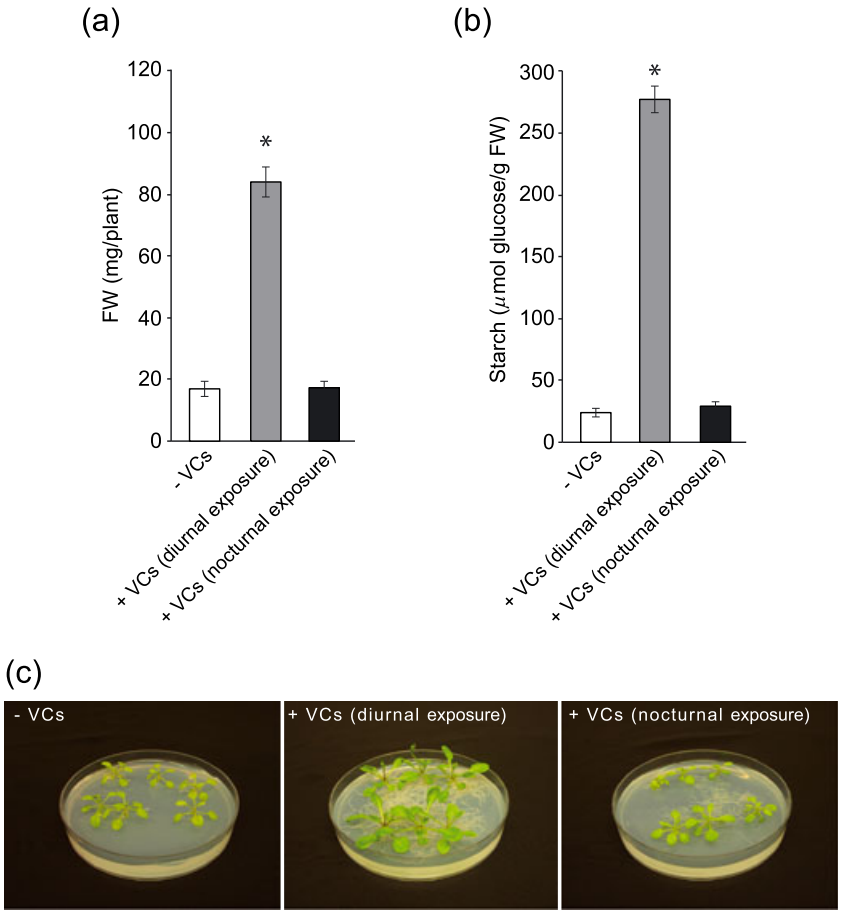

Figure 6. Plant responses to *A. alternata* VCs are light-dependent. (a) FW of rosettes, (b) starch content and (c) external phenotypes of plants cultured in the absence or presence of adjacent cultures of *A. alternata* for 1 week, either only during the light or only during the dark. In a and b values represent the means ± SE determined from four independent experiments conducted using 12 plants in each experiment. Asterisks indicate significant differences between VC-treated and non-treated plants according to Student's *t*-tests ($P < 0.05$).

Information Table S3; Fig. 7). These proteins have inherently photoprotective properties and play an important role in collecting light quanta to deliver them to the reaction centres, where they are converted into chemical forms of energy (Pascal *et al.* 2005). Thus, VC-promoted enhancement of photosynthesis (Table 1; Fig. 3) is probably at least partially because of increases in levels of light-harvesting proteins (Fig. 8).

A. alternata VC-promoted increase of ETR (Fig. 3) creates conditions for the production of reactive oxygen species (ROS), which may result in photoinhibition and subsequent photooxidative damage, a phenomenon that could be prevented by the accumulation of anthocyanins, carotenoids and ROS scavengers. VCs exerted a positive effect on the expression of genes coding for enzymatic ROS scavengers (Supporting Information Table S3; Fig. 7). Furthermore, VCs exerted a negative effect on the expression of the CK-repressed negative *MYBL2* regulator (Dubos *et al.* 2008) and a positive effect on the expression of a number of anthocyanin biosynthesis-related genes including the CK-induced positive regulators *PAP1*/*MYB75* and *TT8* (Das *et al.* 2012) and structural genes *TT4* and *UF3GT* (Supporting Information Table S3; Fig. 7). Moreover, VCs exerted a negative effect on the expression of the CK-repressed *NCED4* gene involved in carotenoid degradation (Gonzalez-Jorge *et al.* 2013) (Supporting Information Table S3; Fig. 7). Therefore, CK-induced modulation of genes coding for anthocyanins, ROS scavengers and carotenoid content regulators may contribute to the enhancement of photosynthetic capacities observed in VC-treated plants (Fig. 8).

Glucosinolates are sulfur-rich amino acid-derived secondary plant products that act as important determinants of plant growth, development and defence against pathogens (Tantikanjana *et al.* 2001; He *et al.* 2011; Imhof *et al.* 2014). *Alternaria alternata* VCs promoted the expression of a number of glucosinolate biosynthesis-related genes (Supporting Information Table S3; Fig. 7). Some of them (e.g. *IPMI1* and *GSTU20*) are induced by CKs (Brenner & Schmülling 2015). Others (e.g. *CYP79F1*) play important roles in modulating the intracellular levels of CKs (Tantikanjana *et al.* 2004). Thus, CK-promoted up-regulation of glucosinolate biosynthesis-related genes and/or glucosinolate-mediated enhancement of CK levels probably contribute to the VC-promoted early flowering and enhancement of growth.

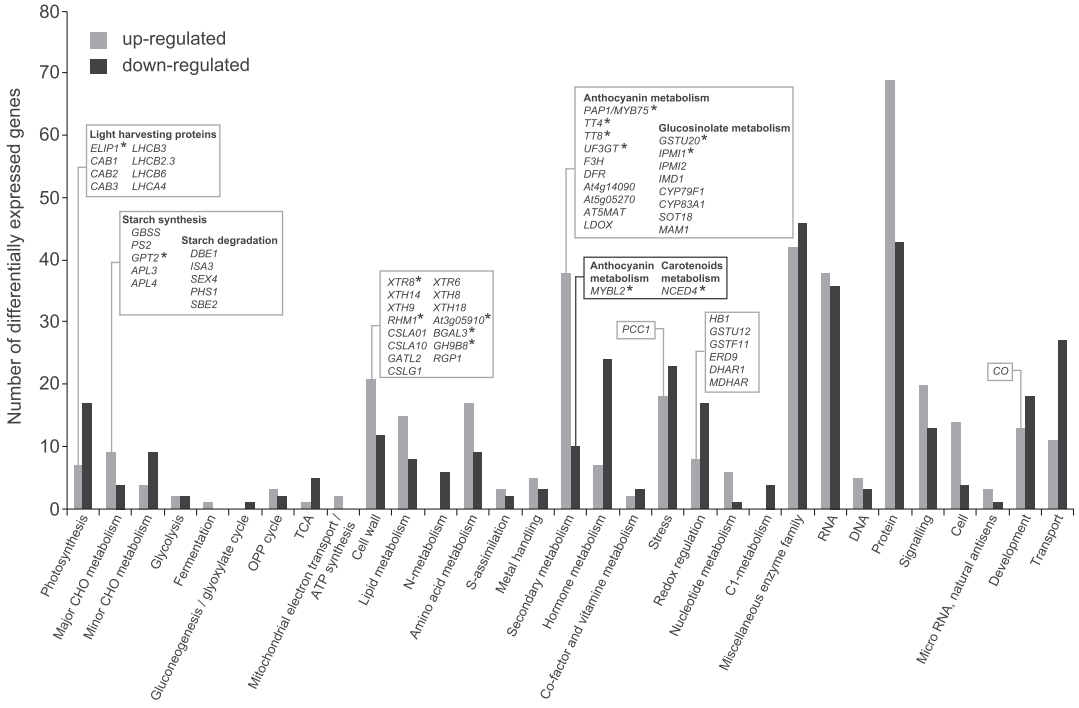

Figure 7. Functional categorization of the transcripts differentially expressed in leaves of *Arabidopsis* plants cultured in the presence of VCs emitted by *A. alternata*. Transcripts were identified using the *Arabidopsis* Gene Expression Microarray 4 × 44 K (G2519, Agilent Technologies). Significantly, down- and up-regulated transcripts in exposed plants, with a 2.0-fold change relative to non-exposed plants, were sorted according to putative functional category assigned by MapMan software. Numbers of up- and down-regulated genes in each categorical group are indicated by grey and black bars, respectively. Genes discussed here are boxed, and CK-regulated genes are indicated with asterisks.

Our transcriptomic analyses revealed that *A. alternata* VCs enhance expression of a number of genes involved in cell wall composition, strength and extensibility (Supporting Information Table S3; Fig. 7). Some of them (e.g. *XTR8*, *RHM1*, *BGAL3*, *GH9B8* and *At3g05910*) are up-regulated by CKs. Because cell wall synthesis and extensibility are major determinants of growth, VC-promoted growth may be at least partly mediated by CK-promoted induction of cell wall-related genes (Fig. 8).

A. alternata VCs also promoted the expression of starch biosynthetic genes, such as those encoding the non-catalytic large subunits of ADPglucose pyrophosphorylase APL3 and APL4, the granule bound starch synthase (GBSS) and inorganic pyrophosphatase (PS2) (Supporting Information Table S3; Fig. 7) and starch-degradation-related genes such as *DBE1*, *ISA3*, *SEX4*, *PHS1* and *SBE2*. As mentioned earlier, a striking alteration in the transcriptome of VC-treated plants involves the strong up-regulation of the CK-induced G6P/Pi transporter encoding *GPT2* gene. Thus, accumulation of exceptionally high levels of starch in leaves of VC-treated plants probably involves CK-induced GPT2-mediated transport of cytosolic G6P, which once in the chloroplast, is metabolized into starch (Fig. 8).

In *Arabidopsis*, the light-controlled *CONSTANS* (*CO*) plays a central role in the regulation of flowering (An *et al.* 2004). Recent studies have shown that *CO*-mediated regulation of *GBSS* and *PCC1* expression is an important element of the induction of floral transition (Segarra *et al.* 2010; Ortiz-Marchena *et al.* 2014). Notably, VCs stimulated the expression of *CO*, *PCC1* and *GBSS* (Supporting Information Table S3; Fig. 7). Therefore, it is tempting to speculate that VC-promoted floral transition involves stimulation of *CO* expression (Fig. 8).

In *Arabidopsis*, nitric oxide (NO) represses floral transition by suppressing *CO* expression (He *et al.* 2004). Furthermore, high concentrations of this gaseous compound inhibit the electron transport activity in PSII and photophosphorylation (Takahashi & Yamasaki 2002). VCs promoted the expression of the non-symbiotic haemoglobin HB1 (Supporting Information Table S3; Fig. 7), which together with CKs acts as scavenger and suppressor of NO action (Perazzolli *et al.* 2006; Liu *et al.* 2013). Furthermore, high levels of HB1 expression promote early flowering and growth (Hunt *et al.* 2002; Hebelstrup & Jensen 2008). Therefore, it is highly conceivable that suppression of NO action contributes to VC-promoted early flowering and enhancement of photosynthesis (Fig. 8).

Plants react to volatile compounds emitted by phylogenetically diverse microorganisms through highly conserved mechanisms involving CK signalling

Plants have evolved the capacity to detect VCs released by a plethora of microorganisms. The findings that mixtures of VCs emitted by all microbial species tested in this work

Table 3. Sets of the 20 most strongly up-regulated and 20 most strongly down-regulated genes in plants exposed to VCs emitted by *B. subtilis* that are also up- and down-regulated by VCs emitted by *A. alternata*

Up-regulated genes	
ID	Description
AT1G61800*	glucose-6-phosphate/phosphate translocator 2 mRNA, complete cds [NM_104862]
AT3G18000	conserved peptide upstream open reading frame 30 mRNA, complete cds [NM_001125181]
AT4G39210	glucose-1-phosphate adenylyltransferase large subunit 3 mRNA, complete cds [NM_120081]
AT5G17220	glutathione S-transferase phi 12 mRNA, complete cds [NM_121728]
AT1G56650	transcription factor MYB75 mRNA, complete cds [NM_104541]
AT2G41090	calmodulin-like protein 10 mRNA, complete cds [NM_129674]
AT4G22870	leucoanthocyanidin dioxygenase-like protein mRNA, complete cds [NM_001160794]
AT1G62560	flavin-containing monooxygenase FMO GS-OX3 mRNA, complete cds [NM_104934]
AT5G48850	protein SULPHUR DEFICIENCY-INDUCED 1 mRNA, complete cds [NM_124262]
AT1G49860	glutathione S-transferase (class phi) 14 mRNA, complete cds [NM_103873]
AT4G22880	leucoanthocyanidin dioxygenase mRNA, complete cds [NM_118417]
AT3G26960*	pollen Ole e 1 allergen and extensin family protein mRNA, complete cds [NM_113610]
AT1G56150	SAUR-like auxin-responsive protein mRNA, complete cds [NM_104494]
AT5G19470	nudix hydrolase 24 mRNA, complete cds [NM_121952]
Down-regulated genes	
ID	Description
AT4G33150	lysine-ketoglutarate reductase/saccharopine dehydrogenase bifunctional enzyme mRNA, complete cds [NM_001160811]
AT4G36850	PQ-loop repeat family protein/transmembrane family protein mRNA, complete cds [NM_119849]
AT5G17300	myb family transcription factor RVE1 mRNA, complete cds [NM_121736]
AT1G53870	TUB_2 domain-containing protein mRNA, complete cds [NM_104264]
AT2G01530	MLP-like protein 329 mRNA, complete cds [NM_126214]
AT1G73750	uncharacterized protein mRNA, complete cds [NM_106034]
AT3G26740**	CCR-like protein mRNA, complete cds [NM_113585]
AT3G49790	Carbohydrate-binding protein mRNA, complete cds [NM_114839]
AT5G56870	beta-galactosidase 4 mRNA, complete cds [NM_125070]
AT4G16690**	methyl esterase 16 mRNA, complete cds [NM_117770]
AT1G75380**	bifunctional nuclease 1 mRNA, complete cds [NM_179559]
AT3G13750	beta galactosidase 1 mRNA, complete cds [NM_112225]
AT1G80920**	chaperone protein dnaJ 8 mRNA, complete cds [NM_106740]
AT5G63160**	BTB and TAZ domain protein 1 mRNA, complete cds [NM_125711]
AT4G35770	senescence-associated protein DIN1 mRNA, complete cds [NM_119743]
AT2G05540	glycine-rich protein mRNA, complete cds [NM_126577]
AT5G49360	bifunctional {beta}-D-xylosidase/{alpha}-L-arabinofuranosidase mRNA, complete cds [NM_124313]

Genes that are up-regulated and down-regulated by CKs are indicated with one and two asterisks, respectively.

promote growth, early flowering and accumulation of exceptionally high levels of starch would indicate that plants respond in a similar manner to diverse microbial VCs. Changes observed in transcriptomes of leaves of *Arabidopsis* plants exposed to VCs emitted by such phylogenetically distant microbial species as the beneficial PGPR *B. subtilis* GB03 (Zhang *et al.* 2007) and fungal plant pathogen *A. alternata* (this work) were strikingly similar (Table 3, Supporting Information Tables S4 and S5). Thus, under appropriate culture conditions at least, many microorganisms (both bacteria and fungi that are beneficial to plants and phyopathogens) can modify plants' physiology and development by triggering highly conserved molecular mechanisms in response to a wide range of VCs. Furthermore, the finding that ~25% of the most differentially regulated genes in plants exposed to VCs emitted by *B. subtilis* and *A. alternata* are CK responsive genes (Table 3) strongly indicates that such molecular mechanisms involve CK signalling. Clearly, further research is needed to identify and characterize the signalling and regulatory mechanisms involved in plants' responses to VCs emitted by different microbial species and to understand their roles in the plant–microbe interactions.

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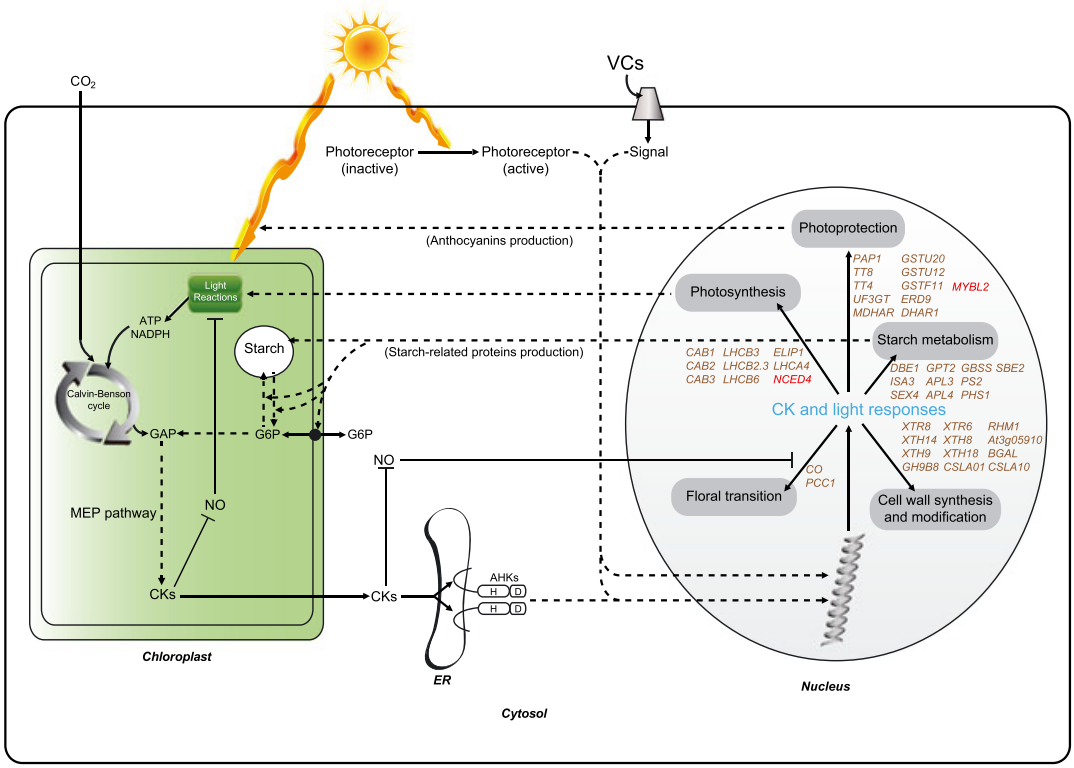

Figure 8. Suggested model for the regulatory network involving CKs and light in response to *A. alternata* VCs. According to this model VCs interact with as yet unidentified plasma membrane receptors to produce signals that rapidly promote changes in the expression of light-induced genes encoding proteins involved in photosynthesis and photoprotection (mainly light harvesting proteins, anthocyanins, ROS scavengers and carotenoids regulators). Augmentation of the photosynthetic activity results in enhanced GAP, which enters the MEP pathway fueling production of plastidic CKs that, once exported to the cytosol and sensed in the ER by AHK receptors, initiate a cascade of reactions resulting in responses such as production of proteins involved in light harvesting and photoprotection, cell wall modification, initiation of floral transition and GPT2-mediated transport of G6P from cytosol to chloroplast. G6P incorporated into the chloroplast is utilized for production of starch and/or CKs. According to this suggested model, VC-promoted early flowering and enhancement of photosynthesis involve suppression of NO action through the scavenging of NO molecules by CKs and HB1. Genes up- and down-regulated by *A. alternata* VCs are indicated in brown and red, respectively.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Photographs illustrating the system for exposing plants to *A. alternata* volatile compounds (VCs) used in this study. Exposure systems for investigating effects of *A. alternata* VCs on (a) *Arabidopsis* plants cultured in MS medium and (b, c) maize and pepper plants cultured in soil. Plants were cultured in the absence or continuous presence of adjacent microbial cultures with no physical contact.

Figure S2. VCs emitted by phylogenetically diverse microorganisms promote accumulation of exceptionally high levels of starch in *Arabidopsis* leaves. Starch contents in leaves of illuminated plants cultured in the absence or continuous presence of adjacent cultures of the indicated microorganisms for 1 day. Values represent the means ± SE determined from four independent experiments using 12 plants in each experiment. Asterisks indicate significant differences between VC-treated and control (non-treated) plants based on Student's *t*-tests ($P < 0.05$). The phylogenetic tree was constructed using the PhyloT phylogenetic tree generator (www.phyloT.biobyte.de).

Figure S3. VCs emitted by *A. alternata* promote augmentation of the levels of CKs in *Arabidopsis* leaves. Scheme representing pathways of CK biosynthesis through the plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP) and cytosolic mevalonate (MVA) pathways in leaves of VC-treated plants. Black arrows show the biosynthesis, interconversions and metabolic flow of CKs in *Arabidopsis* cell (adapted from Spíchal 2012). Multistep reactions are depicted with hollow arrows. The green arrows indicate a hypothetical exchange of common precursor(s) between the MEP and MVA pathways (adapted from Kasahara *et al.* 2004). Metabolites whose levels are enhanced by VCs (cf. Table 2) are highlighted in blue. CKs whose levels are decreased by VCs (cf. Table 2) are highlighted in red. iPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate.

Figure S4. Relative abundance of transcript levels in leaves of illuminated *Arabidopsis* plants in the presence of VCs emitted by *A. alternata*. Fold change values represent changes in levels

of transcripts (measured by quantitative RT-PCR) in leaves of plants cultured in the presence of VCs and harvested at the end of the light period for 16 h, relative to those of control leaves of plants cultured in the absence of VCs. Primers used are listed in Supporting Information Table S2.

Table S1. Microorganisms used in this study

Table S2. Primers used in qRT-PCR

Table S3. List of genes whose expression is altered by *A. alternata* VCs treatment. Genes that are up-regulated by CKs are highlighted in blue colour. Genes that are down-regulated by CKs are highlighted in yellow colour (Tantikanjana *et al.* 2004; Das *et al.* 2012; Bhargava *et al.* 2013; Brenner & Schmülling 2012, 2015)

Table S4. List of genes whose expression is down-regulated by VCs emitted by *A. alternata* (this work, cf. Supporting Information Table S3) and by *B. subtilis* GB03 (cf. Supporting Information Table S1 in Zhang *et al.* 2007)

Table S5. List of genes whose expression is up-regulated by VCs emitted by *A. alternata* (this work, cf. Supporting Information Table S3) and by *B. subtilis* GB03 (cf. Supporting Information Table S1 in Zhang *et al.2007*)