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Original Article

Petunia × *hybrida* floral scent production is negatively affected by high-temperature growth conditions

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ABSTRACT

Increasing temperatures due to changing global climate are interfering with plant–pollinator mutualism, an interaction facilitated mainly by floral colour and scent. Gas chromatography–mass spectroscopy analyses revealed that increasing ambient temperature leads to a decrease in phenylpropanoid-based floral scent production in two *Petunia* × *hybrida* varieties, P720 and Blue Spark, acclimated at 22/16 or 28/22 °C (day/night). This decrease could be attributed to down-regulation of scent-related structural gene expression from both phenylpropanoid and shikimate pathways, and up-regulation of a negative regulator of scent production, emission of benzenoids V (EOBV). To test whether the negative effect of increased temperature on scent production can be reduced in flowers with enhanced metabolic flow in the phenylpropanoid pathway, we analysed floral volatile production by transgenic 'Blue Spark' plants overexpressing CaMV 35S-driven *Arabidopsis thaliana production of anthocyanin pigments 1* (*PAP1*) under elevated versus standard temperature conditions. Flowers of *35S:PAP1* transgenic plants produced the same or even higher levels of volatiles when exposed to a long-term high-temperature regime. This phenotype was also evident when analysing relevant gene expression as inferred from sequencing the transcriptome of *35S:PAP1* transgenic flowers under the two temperature regimes. Thus, up-regulation of transcription might negate the adverse effects of temperature on scent production.

Key-words: anthocyanin; environmental stimulus; petunia; phenylpropanoid; production of anthocyanin pigment1 (PAP1); temperature; volatile.

INTRODUCTION

During evolution, plants have adapted to an array of habitats with extremely diverse environmental conditions. As sessile organisms that are often subjected to both diel and seasonal temperature shifts, plants have developed complex mechanisms for sensing and responding to environmental cues (Kotak *et al*. 2007). The ability to survive in extreme conditions through priming and plasticity in adjusting to temperature changes is one of the major parameters contributing to plant fitness (Penfield 2008; McClung & Davis 2010).

Ambient temperatures affect cell growth, elongation and division (Tardieu & Granier 2000), synchronization of the circadian clock (Salome & McClung 2005), flowering time and intensity, fruit set (Halliday & Whitelam 2003) and overall yield (Lobell & Asner 2003). Despite significant progress in uncovering the molecular mechanisms by which plants perceive and mediate temperature cues, these processes are still far from being completely understood (Mittler *et al*. 2011).

The average global temperature has increased in the past century, with the rate of increase accelerating over the last 60 years. It has been reported that for the northern hemisphere, the period between 1983 and 2012 has likely been the warmest 30-year period in the last 1400 years (Stocker *et al*. 2013). In addition, over the past four decades, spring events have advanced by 2.5 days per decade (Menzel *et al*. 2006), whereas heat-wave episodes and warmer nights have become more frequent (Stocker *et al*. 2013). These changes in global climate have already affected plant–pollinator mutualism because of temporal or spatial mismatches in these interactions (Gordo & Sanz 2005; Hegland *et al*. 2009; Kroner & Basler 2010; Kudo & Ida 2013).

Plant–pollinator interactions are facilitated by floral architecture, colour and scent (Hoballah *et al*. 2007; Klahre *et al*. 2010). Floral scent represents an elaborate phenotype determined by a complex mix of volatile compounds (or in some cases a single compound), which through its temporal manifestation allows plant coordination with its pollinator (Hoballah *et al*. 2005; Oyama-Okubo *et al*. 2005). Floral volatile compounds can be roughly divided into three main classes based on their biosynthetic origin: fatty acid derivatives, isoprenoids and phenylpropanoids (Pichersky *et al*. 2006). The latter are derived from the amino acid phenylalanine and can be further divided into three subclasses: benzenoid (with a C6–C1 carbon backbone), phenylpropanoid-related (C6–C2) and phenylpropene (C6–C3) compounds. The first committed step in benzenoid and phenylpropanoid biosynthesis is

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catalysed by L-phenylalanine ammonia lyase (PAL) (Boatright *et al*. 2004), which converts L-phenylalanine to *t*-cinnamic acid. Cinnamate 4-hydroxylase (C4H) (Anterola *et al*. 2002) diverts carbon from *t*-cinnamic acid to phenylpropene compounds, whereas benzenoids are formed by side chain shortening of *t*-cinnamic acid via a β-oxidative and/or non-β-oxidative pathway. Phenylpropanoid-related compounds are directly produced from phenylalanine by the action of phenylacetaldehyde synthase (PAAS) (Kaminaga *et al*. 2006; Long *et al*. 2009; Vogt 2009; Muhlemann *et al*. 2014). In the last few years, several gene-encoding enzymes that yield specific end products of the pathway have been identified (Negre *et al*. 2003; Boatright *et al*. 2004; Koeduka *et al*. 2006, 2008; Farhi *et al*. 2010). A better understanding of the transcriptional network regulating the phenylpropanoid pathway has been the focal point of several recent studies, resulting in the characterization of a handful of transcriptional regulators belonging to the R2R3–MYB family. Although ODORANT1 coordinates the transcription of genes in the shikimate pathway, emission of benzenoids I and II (EOBI and EOBII) positively regulate the expression of genes in the shikimate as well as phenylpropanoid pathways (Verdonk *et al*. 2005; Spitzer-Rimon *et al*. 2010, 2012; Colquhoun & Clark 2011; Van Moerkercke *et al*. 2011). MYB4, which targets *PhC4H*, and emission of benzenoids V (EOBV), whose direct targets are still unknown, are negative regulators of the phenylpropanoid pathway (Colquhoun *et al*. 2010a; Spitzer-Rimon *et al*. 2012). The *Arabidopsis thaliana* R2R3–MYB production of anthocyanin pigment-1 (*AtPAP1*) is another example of a transcriptional regulator of floral scent. Ectopic expression of *AtPAP1* in petunia and rose flowers enhances both colour and scent production by activating a number of genes in the phenylpropanoid pathway (Borevitz *et al*. 2000; Zvi *et al*. 2008, 2012).

It is well established that the phenylpropanoid pathway is regulated by environmental conditions, for example, high-temperature growth conditions lead to down-regulation of *PAL* isoforms and decreased anthocyanin pigmentation in flowers (Christie *et al*. 1994; Shaked-Sachray *et al*. 2002; Olsen *et al*. 2008; Rowan *et al*. 2009). Unlike anthocyanins, data regarding floral volatile phenylpropanoid production under various temperatures are still limited. Several studies aimed at understanding the emission pattern of such compounds concluded that an elevation in ambient temperatures results in their increased emission and diminished levels in endogenous pools. For example, Jakobsen & Olsen (1994) reported that *Trifolium repens* L. flowers grown at 15 °C emit more volatile phenylpropanoids when briefly exposed to 20 °C and less when exposed to 10 °C. Sagae *et al*. (2008) concluded that the highest level of emission by *Petunia axillaris* flowers grown at continuous ambient temperatures of 20, 25, 30 and 35 °C (measured at night at a single point in the life of the flower) was at 30 °C, whereas the internal pool continued to decrease as ambient temperature rose.

In this study, we examined the effect of increasing temperature on floral volatile production and emission in *Petunia* × *hybrida*, a well-established system for scent studies. The observed decrease in scent production/emission coupled with temperature increase from 22/16 to 28/22 °C (day/night) was partially attributed to down-regulation of scent-related gene expression. We further demonstrate that transcriptional up-regulation of the phenylpropanoid pathway via ectopic expression of *AtPAP1* can reduce the adverse effect of high temperature on floral scent.

MATERIALS AND METHODS

Plant material

Rooted petunia plantlets (*Petunia* × *hybrida* line P720, cv. Blue Spark and *35S:PAP1*-transgenic Blue Spark) were obtained from Danziger – 'Dan' Flower Farm (Mishmar Hashiva, Israel). Plants were initially grown under standard conditions (22/16 °C day/night, 16/8 h light/dark photoperiod) for a month, and then transferred to either 28/22 or 34/28 °C day/night (for line P720 only) for another month, unless otherwise specified.

Collection and gas chromatography–mass spectrometry (GC–MS) analysis of volatile compounds

For dynamic headspace analysis (Spitzer-Rimon *et al*. 2012), flowers were collected 1 day post-anthesis (dpa) at 1100 h. Volatiles emitted from detached petunia flowers (three flowers per sample, with three to seven independent experiments per treatment) were collected for 24 h using an adsorbent trap consisting of a glass tube containing 100 mg of Porapak Type Q polymer (80/100 mesh; Alltech) and 100 mg of charcoal-activated 20/40 mesh (Supelco) held in place with steel mesh plugs. Trapped volatiles were eluted using 1.5 mL hexane, and 2 μg of *iso*butylbenzene was added to each sample as an internal standard. A calibration plot with increasing *iso*butylbenzene concentrations was generated to determine the relative amounts of target volatiles within each sample. To determine the pool sizes of volatile compounds in the corolla limbs (Spitzer-Rimon *et al*. 2012), petal tissues (0.5 g fresh weight, five to eight independent experiments per treatment) were collected at either 0800 or 2000 h (1 dpa), ground in liquid nitrogen and extracted in hexane (4 mL g^{-1} tissue) containing 2 μg of *iso*butylbenzene as the internal standard. Following a 2 h incubation with shaking at 150 r.p.m., extracts were centrifuged at 10 500 *g* for 10 min, and the supernatant was further centrifuged and evaporated prior to chromatography. GC–MS analysis (1 μL sample) was performed using a device composed of a Pal autosampler (CTC Analytic), a TRACE GC 2000 equipped with an Rtx-5SIL mass spectrometer fused-silica capillary column (Restek; i.d. 0.25 μm, 30 m × 0.25 mm) and a TRACE DSQ quadruple mass spectrometer (ThermoFinnigan). Helium was used as the carrier gas at a flow rate of 0.9 mL min^{-1}. The injection temperature was set to 250 °C (splitless mode) and the interface to 240 °C, and the ion source was adjusted to 200 °C. The analysis was performed under the following temperature program: 2 min of isothermal heating at 40 °C followed by a 10 °C min^{-1} oven temperature ramp to 250 °C.

The system was equilibrated for 1 min at 70 °C before injection of the next sample. Mass spectra were recorded at 3.15 scan s^{-1} with a scanning range of 40–450 mass-to-charge ratio and electron energy of 70 eV. Compounds were tentatively identified (>95% match) based on NIST/EPA/NIH Mass Spectral Library data version NIST 05 (software version 2.0d) using the XCALIBUR v1.3 (ThermoFinnigan). Further identification of major compounds was based on comparison of mass spectra and retention times with those of authentic standards (Sigma-Aldrich) analysed under similar conditions.

Real-time quantitative PCR (qPCR) analysis

Petunia total RNA was extracted from 100 mg of flowers (three to nine independent samples per treatment) with the Tri-Reagent kit (Molecular Research Center) and treated with RNase-free DNaseI (Fermentas, Vilnius, Lithuania). First-strand cDNA was synthesized using 1 μg of total RNA, oligo(dT) primer and reverse transcriptase ImProm-II (Promega, Madison, WI, USA). Real-time qPCR was performed (in triplicate for each biological repeat) as described by Spitzer-Rimon *et al*. (2010) for 40 cycles (94 °C for 15 min and then cycling at 94 °C for 10 s, 60 °C for 30 s and 72 °C for 20 s) in the presence of Absolute Blue qPCR SYBR Green ROX Mix (Thermo Fisher Scientific) on a Qiagen Rotor-Gene Q cycler. A standard curve was generated for each gene using dilutions of cDNA samples, and data analysis was performed using Rotor-Gene Q series software 2.1.0. PCR primers used for amplification of gene-specific regions are listed in Supporting Information Table S1. Primer specificity was determined by melting curve analysis; a single, sharp peak in the melting curve ensured that a single, specific DNA species had been amplified.

Anthocyanin contents

To determine anthocyanin levels, petunia corollas were extracted with methanol containing 1% (v/v) HCl (50 mg of fresh tissue per 1 mL of acidic methanol). Absorption values of the extract at 530 and 657 nm were measured using the formula $A_{530} - 0.25(A_{657})$, which allows for subtraction of chlorophyll interference. Four biological replicates per treatment were used for analyses of anthocyanin levels.

Transcriptome analysis

RNA was isolated from corollas of PAP1-overexpressing 'Blue Spark' petunia plants grown at 22 and 28 °C using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA) (two biological replicates per treatment). Libraries were constructed using the TruSeq RNA sample preparation kit (Illumina) essentially as directed by the manufacturer but with the following modifications. Fragmentation time in the elute fragment prime step was reduced from 8 to 4 min, resulting in cDNA ranging in length from 100 to 1000 bp. Subsequent AMPure purification was performed at a sample-to-AMPure ratio of 1.8:1 (v/v). The resulting libraries were composed of amplicons largely ranging in length from 200 to 1000 bp. The libraries were sequenced on an Illumina HiSeq2000 using SBS v2 reagents to produce 100-base paired-end reads. Assembly of reads was performed using Trinity software (Haas *et al*. 2013) and transcript assemblies were annotated for function using Blast2GO (Conesa *et al*. 2005).

Library construction generated a total of 498,493,968 reads. These reads were subjected to quality and adapter trimming, which removed 250,862,102 (50.3%) reads before final *de novo* assembly. The assembly resulted in a total of 227,481 contigs with an N50 of 932 bp. Counts for 227,444 contigs were used for downstream statistical analysis.

Analysis of differentially expressed genes was performed using R and the Bioconductor package EdgeR (Reimers & Carey 2006; Robinson *et al*. 2010). Unique reads with less than one count per million for at least two libraries were filtered prior to identification of differentially expressed genes. Gene ontology (GO) term enrichment analysis was carried out using Blast2GO.

Sequences of biosynthetic genes involved in the benzenoid/phenylpropanoid network and deposited in NCBI were used to identify contigs mapping to these genes. Nucleotide identities higher than 95% and matching sequence lengths longer than 50 nucleotides were used as threshold parameters for contig annotation.

RESULTS

Effect of ambient temperature on floral scent

To characterize the effect of ambient temperatures on floral scent production, rooted plantlets of *Petunia* × *hybrida* line P720 were first grown until flowering (about 1 month) under standard growth conditions (22/16 °C day/night, natural photoperiod) and then transferred to two higher temperature regimes (28/22 and 34/28 °C day/night) for 1 month or left to grow at 22/16 °C day/night. Total volatile emission from flowers at 1 dpa was assayed by dynamic headspace analysis for 24 h followed by GC–MS and compared between temperature regimes. The highest levels of volatiles were emitted by flowers under standard growth conditions (22/16 °C day/night). Levels of floral volatiles emitted by plants grown at either 28/22 or 34/28 °C were ~40% of those emitted by plants grown at 22/16 °C (Fig. 1a). Because no significant differences were observed in the emission levels between the two elevated temperature treatments, we further focused on the effects at 28/22 °C. Analyses of anthocyanin levels in the corollas of plants grown at 22/16 and 28/22 °C revealed the expected (Shaked-Sachray *et al*. 2002; Dela *et al*. 2003) decrease in pigment levels with the rise in temperature (Fig. 1a, inset).

A negative effect of temperature increase from 22/16 to 28/22 °C on floral volatile emission was evident with most analysed phenylpropanoid scent compounds. The levels of emitted benzaldehyde, benzyl alcohol, phenylethyl alcohol, benzyl acetate, benzylbenzoate, eugenol and phenylacetaldehyde were significantly lower in corollas at 28/22 versus 22/16 °C (Fig. 1b). The levels of *iso*eugenol and

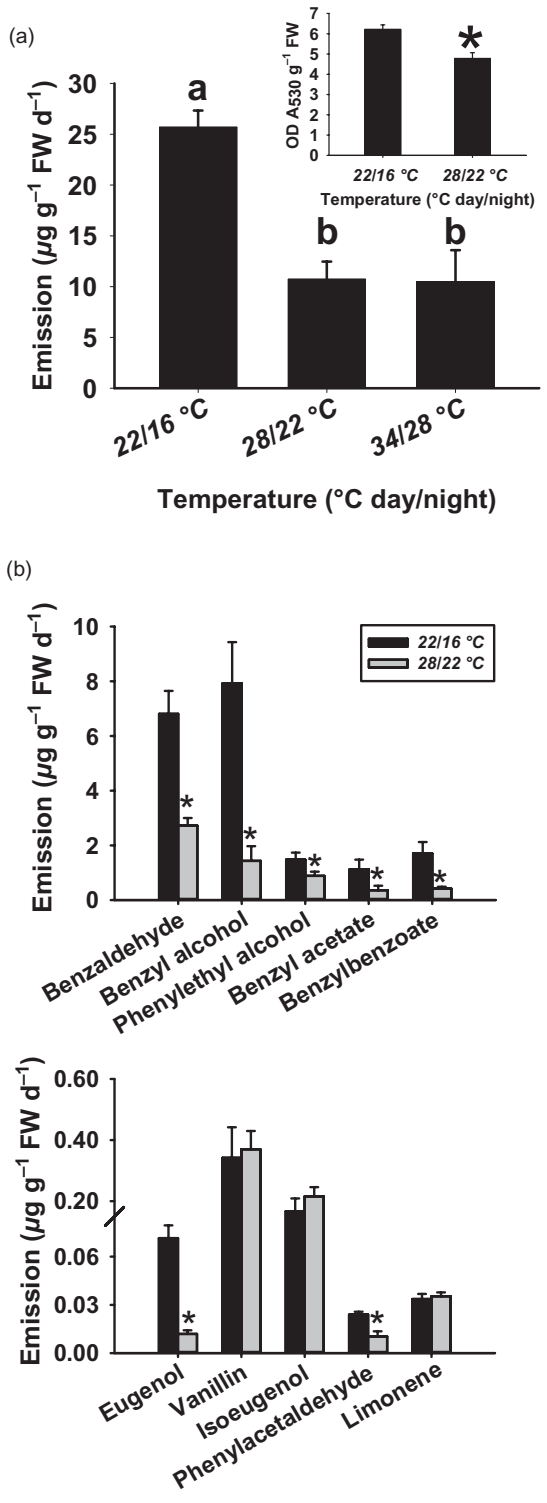

Figure 1. Elevated growth temperature regime reduces the levels of emitted volatile phenylpropanoids and anthocyanins in *Petunia × hybrida* line P720. (a) Dynamic headspace analyses of total emitted volatiles followed by gas chromatography–mass spectrometry (GC–MS) were performed for 24 h on corollas (1 dpa from 1100 h) of petunia plants grown at either 22/16, 28/22 or 34/28 °C day/night. Average values of five to seven independent experiments are shown, with SEs indicated by vertical lines. The significance of the differences in emission levels between temperature regimes was calculated using Tukey's all pairwise multiple comparison procedure following one-way analysis of variance. Values with different letters are significantly different at $P < 0.05$. Inset: anthocyanin content in corollas. Values are averages of four independent experiments with SEs indicated by vertical lines. Significance of differences between treatments was calculated using Student's *t*-test: $*P \leq 0.05$. (b) Dynamic headspace analyses of individual scent compounds using GC–MS were performed on corollas (1 dpa for 24 h from 1100 h) of petunia plants grown at either 22/16 or 28/22 °C day/night. Values are averages of five to seven independent experiments with SEs indicated by vertical lines. Significance of differences between treatments was calculated using Student's *t*-test: $*P \leq 0.05$.

vanillin, as well as the terpenoid limonene, were not affected by the change in temperature (Fig. 1b).

An analysis of internal pool sizes of volatiles in corollas of plants grown at high temperature revealed that they were also strongly affected by the rise in temperature: the level of all phenylpropanoid scent compounds in the morning, except vanillin, was significantly lower (up to 90% decrease, Fig. 2a) under 28/22 versus 22/16 °C day/night conditions. The decrease in the levels of volatiles accumulated by corollas at 28/22 °C was evident for most volatiles at both time points analysed, 0800 and 2000 h, when pools are at their lowest and highest levels, respectively (petunia scent emission occurs mostly at dusk and at night). In the evening hours, that is, 2000 h, the levels of the *iso*eugenol and vanillin pools remained unaffected by the temperature increase similar to their emitted levels. The pool levels of the volatile terpenoids limonene and beta-ionone remained the same regardless of changes in temperature (Fig. 2a,b).

The effect of temperature on floral scent was also evaluated in cv. Blue Spark, a petunia variety that was previously studied with regard to transcriptional up-regulation of genes involved in the synthesis of anthocyanin pigments and scent production in the corollas (Zvi *et al.* 2008). Similar to line P720, emission levels of benzaldehyde decreased sharply in flowers of plants grown for 1 month under high versus standard temperature conditions (28/22 versus 22/16 °C day/night). Methyl benzoate, the other constituent of the Blue Spark scent bouquet, was not significantly affected by the temperature shift (Fig. 3a). GC–MS analyses of internal pools of volatiles in corollas of cv. Blue Spark collected at 2000 h 1 dpa revealed significantly lower levels of benzaldehyde, benzyl alcohol, phenylacetaldehyde and eugenol (~33–61% decrease), but not vanillin, benzyl benzoate or methyl benzoate at 28/22 °C compared with standard growth conditions (Fig. 3b). Anthocyanin levels were also strongly reduced in flowers of plants subjected to the 28/22 °C treatment compared with standard temperature conditions (Fig. 3c). It should be noted that as opposed to the afore-presented long-term acclimation of plants to increased temperature, short exposure of flowers to high temperature – plants grown at 22/16 °C and only flowers exposed to 28 versus 22 °C for 8 h during headspace

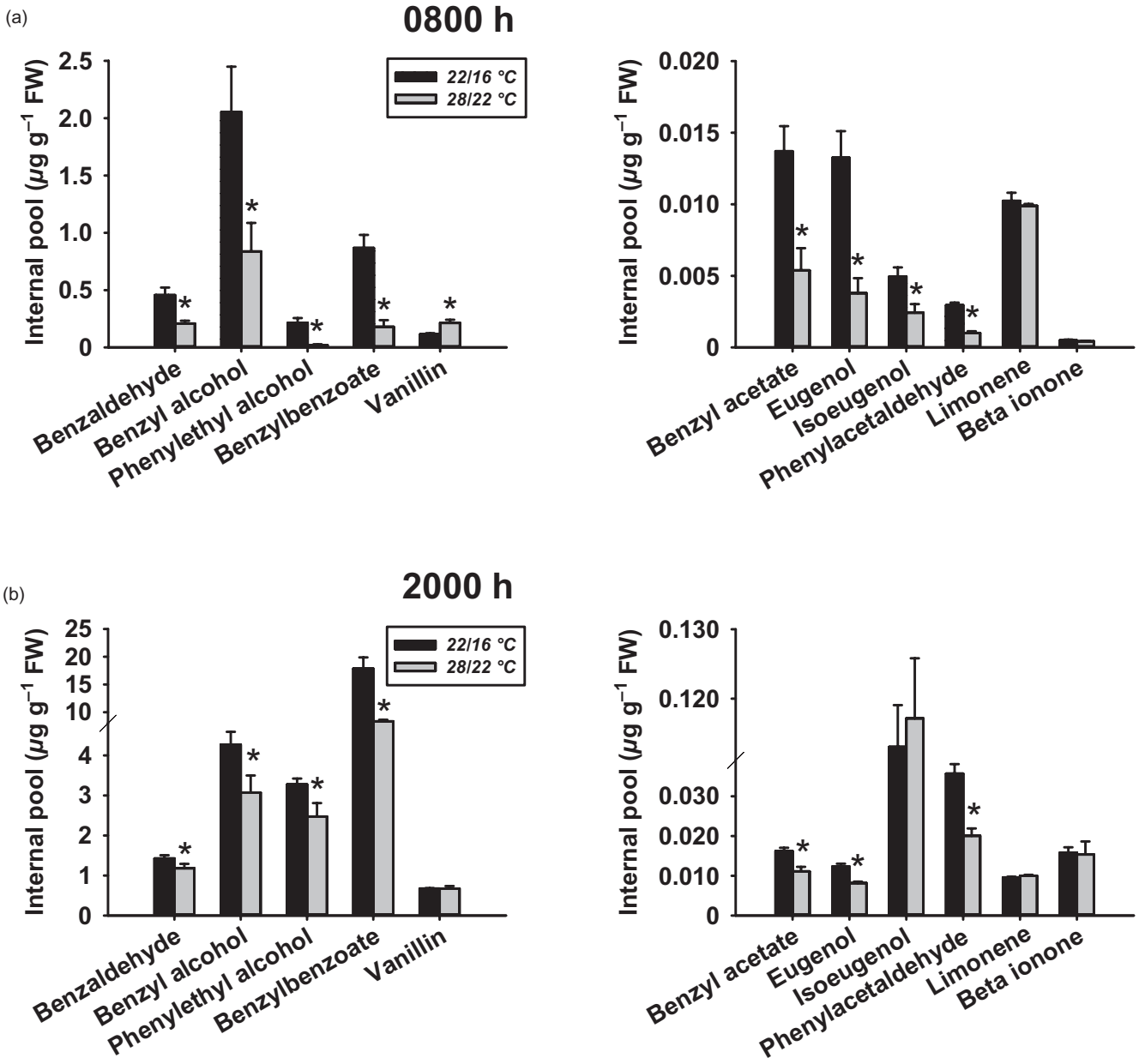

Figure 2. Elevated growth temperature regime induces a decrease in the levels of internal pools of volatile phenylpropanoids in corollas of petunia line P720. Plants were grown at either 22/16 or 28/22 °C day/night and volatiles were sampled for gas chromatography–mass spectrometry (GC–MS) analysis from corollas at 0800 h (a) or 2000 h (b) 1 dpa. Columns represent the mean values of five to eight independent experiments. SEs are indicated by vertical lines. Significance of differences between treatments was calculated using Student's *t*-test: $*P \leq 0.05$.

analyses – led to enhanced emission of benzaldehyde (Supporting Information Fig. S1).

Expression profiling of scent-related genes under different temperature conditions

To examine the expression patterns of scent-related genes in flowers of both 'P720' and Blue Spark petunia plants after 1 month acclimation to 28 versus 22 °C temperature regimes, real-time qPCR analyses of transcripts encoding enzymes and regulatory factors involved in phenylpropanoid production were performed. In line P720, transcript levels of genes encoding specific 'end product' enzymes catalysing the final steps of scent compound biosynthesis [namely phenylacetaldehyde synthase (*PhPAAS*), benzyl CoA:benzyl alcohol/phenylethanol benzoyltransferase (*PhBPBT*) and eugenol synthase (*PhEGS*)] were reduced by 41–44% at higher ambient temperature (Fig. 4). Transcript levels of

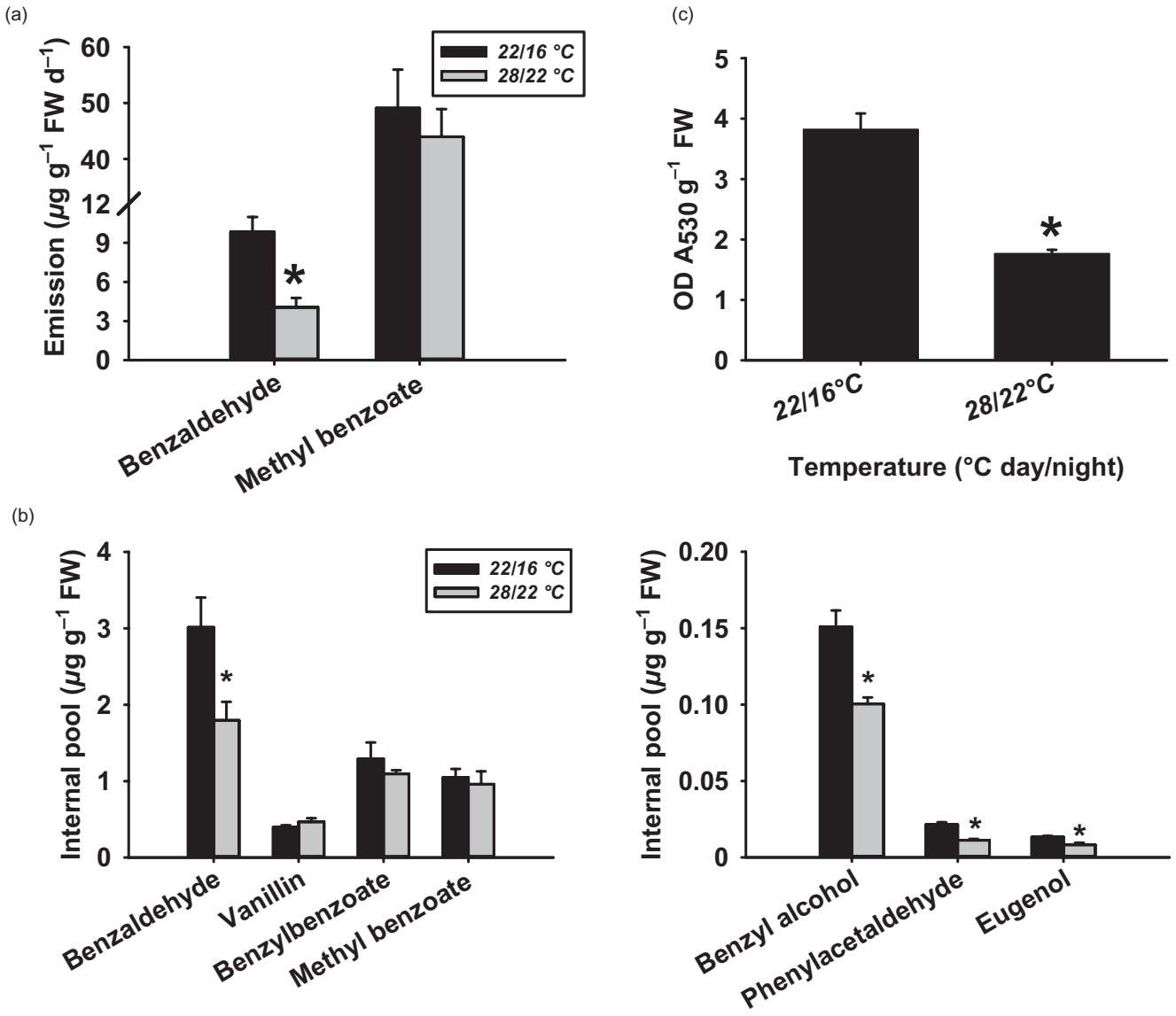

Figure 3. Marked decrease in scent emission, internal pool levels and anthocyanin accumulation in corollas of petunia cv. Blue Spark grown at 28/22 versus 22/16 °C. Dynamic headspace (a) and anthocyanin content (c) analyses were performed as in legend to Fig. 1. Internal pools of individual volatile compounds (b) were assayed as in Fig. 2.

*iso*eugenol synthase (*PhIGS*) were not affected by high temperature treatment, consistent with the lack of change in *iso*eugenol's emission levels (Figs 1 & 4).

To test whether the reduction in scent production under high temperature could also be due to reduced substrate availability, the expression of gene-encoding enzymes involved in the production of precursors of the phenylpropanoid pathway was examined. Levels of transcripts encoding structural enzymes sustaining the metabolic flux toward different branches of the phenylpropanoid pathway, namely coniferyl alcohol acetyltransferase (*PhCFAT*), phenylalanine ammonia lyase (*PhPAL*), 4-coumarate:CoA ligase (*Ph4CL*), cinnamate 4-hydroxylase (*PhC4H*), 3-ketoacyl-CoA thiolase-1 (*PhKAT*) and cinnamic acid:CoA ligase (*PhCNL*), were down-regulated by ~35–50% under 28/22 versus 22/16 °C day/night conditions (Fig. 4). Transcript levels of genes in the shikimate pathway leading to phenylalanine biosynthesis, that is, 5-enolpyruvylshikimate-3-phosphate synthase (*PhEPSPS*), arogenate dehydratase (*PhADT1*), 3-deoxy-D-arabino-heptulosonate 7-phosphate (*PhDAHPS*), chorismate synthase (*PhCS*), prephenate aminotransferase (*PhPPA-AT*) and chorismate mutase (*PhCM*), were also significantly lower in flowers at high temperature (Fig. 4). For comparison, transcript levels of the housekeeping genes *Elongation Factor 1-alpha* (*PhEF1-alpha*) and *Cyclophilin* (*PhCYP*) under the two temperature treatments were essentially the same (Fig. 4). Similar to 'P720', in cv. Blue Spark, transcript levels of gene-encoding enzymes from the shikimate pathway, as well as structural and end product enzymes, were reduced by ~40–70%, with the

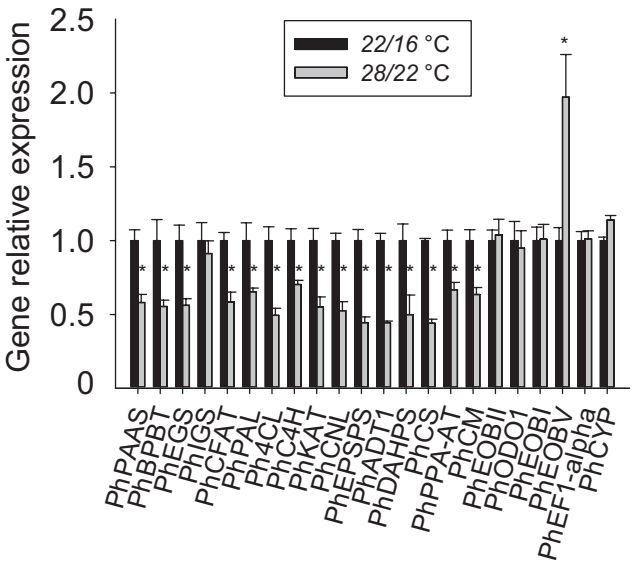

Figure 4. Elevated growth temperature regime leads to down-regulation of scent-related gene expression in petunia line P720 quantitative real-time PCR analyses of *PhPAAS*, *PhBPBT*, *PhEGS*, *PhIGS*, *PhCFAT*, *PhPAL*, *Ph4CL*, *PhC4H*, *PhKAT*, *PhCNL*, *PhEPSPS*, *PhADT1*, *PhDAHPS*, *PhCS*, *PhPPA-AT*, *PhCM*, *PhEOBII*, *PhODO1*, *PhEOBI*, *PhEOBV*, *PhEF1-alpha* and *PhCYP* transcript levels in corollas of petunia plants grown at either 22/16 or 28/22 °C day/night. Samples were collected from corollas 1 dpa at 1300 h. Presented data were normalized to that of 22/16 °C day/night, with SEs indicated by vertical lines. Significance of differences between treatments was calculated by Student's *t*-test based on the raw transcript level data normalized to *PhActin*; $*P \leq 0.05$, $n = 6–9$.

exception of *PhBPBT* and *PhCFAT*, whose expression was not affected by temperature alterations (Fig. 5).

To assess the involvement of scent-related phenylpropanoid pathway-specific transcription factors in mediating the temperature effect, transcript levels of MYB factors *PhEOBI*, *PhEOBII*, *PhODO1* and *PhEOBV* were characterized in both P720 and Blue Spark flowers. In both, expression of *PhEOBI*, *PhEOBII* and *PhODO1*, the key transcriptional activators of the phenylpropanoid and shikimate pathway, was not affected by elevated temperature treatment (Figs 4 & 5). In contrast, transcript levels of the negative regulator *PhEOBV* increased at higher temperature, displaying a trend opposite that observed for most of the structural genes involved in scent production (Figs 4 & 5).

Effect of *AtPAP1* ectopic expression on production of floral volatiles following temperature increase

We previously generated and characterized transgenic *Petunia* × *hybrida* cv. Blue Spark plants expressing the transcription factor *AtPAP1*. In addition to increased pigmentation, these transgenic petunia flowers demonstrate a significant increase in the levels of volatile phenylpropanoid compounds due to increased metabolic flux resulting from transcriptional activation of scent-related genes by *PAP1* (Zvi *et al*. 2008). To test whether the adverse effect of increased temperature on scent production can be negated in flowers with enhanced metabolic flow, we analysed these *35S:PAP1* transgenic flowers following 1 month acclimation to 22/16 versus 28/22 °C day/night temperature regimes. Examination of volatiles present in the internal pools of *35S:PAP1* transgenic corollas revealed that in contrast to the response of control non-transgenic plants, levels of all volatiles were either the same (namely benzaldehyde, benzyl benzoate, benzyl alcohol and phenylacetaldehyde) or higher (eugenol and vanillin) under the high temperature regime (Fig. 6a). Levels of volatiles emitted from the corollas as well as those of anthocyanins accumulated in corollas were not significantly affected by the increase in growth conditions (Fig. 6b).

Gene expression profiling of *35S:PAP1* transgenic plants grown at high temperature versus standard growth conditions

To identify global changes in gene expression in *35S:PAP1*-transgenic cv. Blue Spark flowers of plants grown at 22 versus 28 °C, RNA-Seq analysis was carried out on two biological replicates per temperature condition, consisting of RNA extracted from petals at 1 dpa. On average, 61.9 million reads were generated per sample. These reads were assembled into 227,481 contigs using the Trinity method (Grabherr *et al*. 2011), 34% of which were annotated using Blast2GO (Conesa *et al*. 2005). Prior to the analysis of differential gene

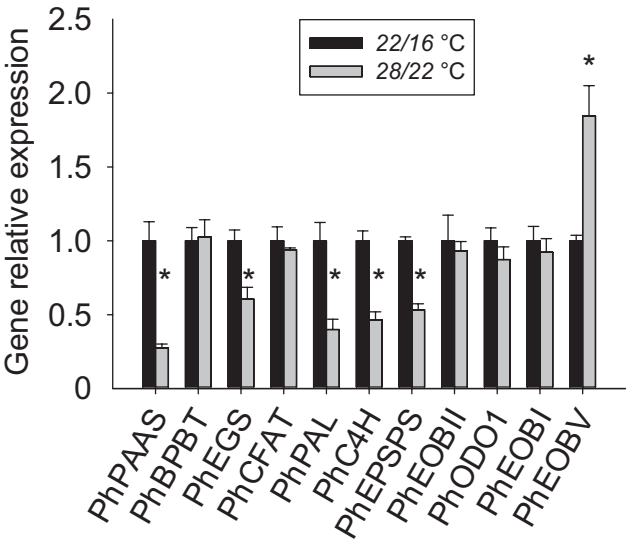

Figure 5. Elevated growth temperature regime leads to a decrease in scent-related gene expression in corollas of petunia cv. Blue Spark. Quantitative real-time PCR analyses of *PhPAAS*, *PhBPBT*, *PhEGS*, *PhCFAT*, *PhPAL*, *PhC4H*, *PhEPSPS*, *PhEOBII*, *PhODO1*, *PhEOBI* and *PhEOBV* transcript levels in corollas of petunia plants grown at either 22/16 or 28/22 °C day/night. Sample collection and analyses were performed as in Fig. 4.

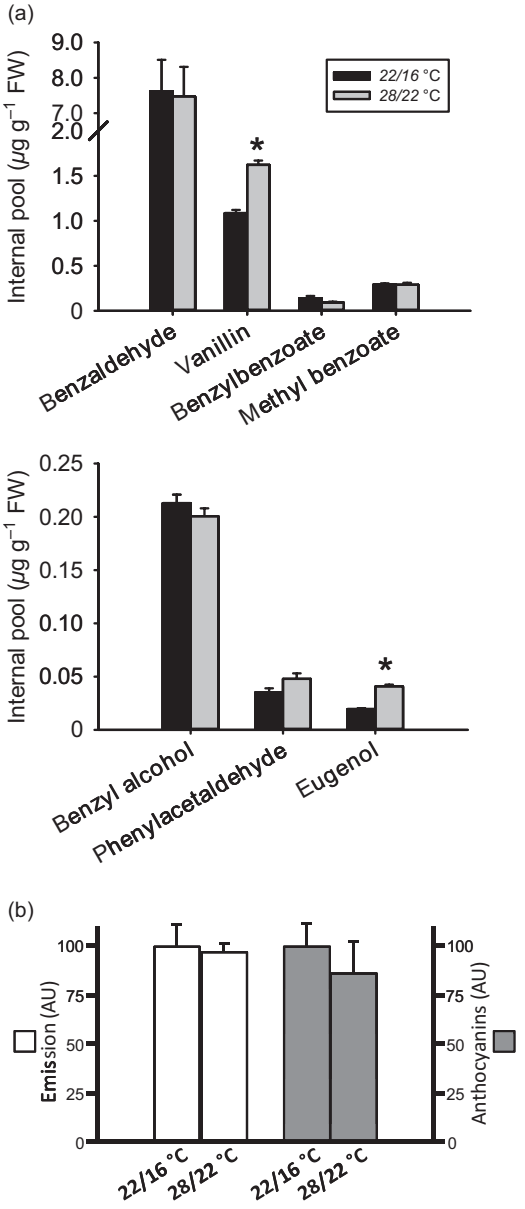

Figure 6. Ectopic expression of *AtPAP1* in petunia cv. Blue Spark negates the effect of high ambient temperature on the levels of volatiles and anthocyanins. Internal pool of accumulated volatiles (a) and emitted volatiles and anthocyanins (b) were assayed as in Fig. 3.

expression, contigs with low count numbers (cut-off was set to one count per million in at least two libraries) were filtered out, resulting in 65,175 contigs. Analysis of differential gene expression revealed that an increase in temperature from 22 to 28 °C leads to reduced abundance of 1286 contigs and increased abundance of 1135 contigs in *35S:PAP1* transgenic petunia flowers.

GO term analysis of down-regulated contigs revealed enrichment of biological processes related to aromatic amino acid catabolism (e.g. GO terms 'tryptophan catabolic process', 'stilbene biosynthetic process', 'coumarin biosynthetic process', 'lignin biosynthetic process', 'indole biosynthetic process'), sugar metabolism (e.g. 'sucrose catabolic process', 'starch catabolic process', 'substituted mannan metabolic process') and response to cold (Table 1). Heat and high-light intensity responses were among the enriched biological processes in the set of up-regulated contigs (e.g. GO terms 'response to heat', 'response to hydrogen peroxide', 'protein unfolding', 'protein refolding', 'response to topologically incorrect protein'). Analyses of the expression levels of individual scent-related genes within the phenylpropanoid network revealed no significant differences in most of the evaluated contigs (e.g. *PhBPBT*, *PhPAL*, *PhBSMT*, *PhC4H*, *PhEGS*, *PhCFAT*, *PhCNL* and *PhKAT*) due to the rise in temperature. Contigs mapping to *PhPAAS*, cinnamoyl-CoA hydratase dehydrogenase (*PhCHD*) and *Ph4CL* were significantly (up to 75%) down-regulated (Fig. 7). To further corroborate the gene expression analyses, real-time qPCR was performed on scent-related transcripts from *35S:PAP1* transgenic flowers. Expression levels of specific 'end product' enzymes *PhBPBT* and *PhEGS* were not significantly affected by the high-temperature regime, whereas the level of *PhPAAS* transcript decreased by ~50% (Fig. 8). Accumulation of transcripts encoding structural enzymes in the phenylpropanoid pathway was also not significantly affected (*PhPAL*, *PhC4H* and *PhCFAT*). The effect of temperature on transcriptional regulators in *35S:PAP1* transgenic flowers was essentially the same as that in non-transgenic Blue Spark plants: real-time qPCR analyses of RNA levels of the negative regulator *PhEOBV* revealed its up-regulation, whereas levels of the activators *PhEOBII*, *PhODO1* and *PhEOBI* were not significantly affected by high temperature in the *35S:PAP1* background (Fig. 8).

DISCUSSION

Scent and colour are key traits for attracting pollinators to flowers, thus allowing sexual reproduction and gene transfer within and among plant populations. Pollination by animals contributes to the sexual reproduction of over 90% of all known angiosperms (Kearns *et al*. 1998) and is essential in 39 of the 57 leading individual crops that altogether represent about 35% (23×10^8 Mt) of global food production (Klein *et al*. 2007). Insect pollinators are sensitive to ambient temperatures (Stone & Stone 1993; Bishop & Armbruster 1999), with elevated temperatures limiting their activity, especially at high altitude (Kjøhl *et al*. 2011). Moreover, recent changes in global climate have already had a documented effect on pollinator behaviour, distribution and interaction with their plant counterparts (Gordo & Sanz 2005; Hegland *et al*. 2009; Kudo & Ida 2013). The effect of temperature on the production of floral scent, one of the key attractants for pollinators, is not well documented. Here we show that when petunia plants are grown under a high temperature regime (28/22 °C day/night) there is a general decrease in the levels of emitted and internal pool volatiles.

Only a handful of studies regarding the effect of environmental conditions on floral volatile phenylpropanoid production have been performed. The most comprehensive work on this subject was conducted by Sagae *et al*. (2008), who

Table 1. List of enriched GO biological processes in contigs down- or up-regulated upon exposure to 28 °C in *35S:PAP1* petunia flowers

Category	GO term	Description	FDR
Down-regulated	GO:0015689	Molybdate ion transport	8.23×10^{-07}
	GO:0009811	Stilbene biosynthetic process	2.14×10^{-06}
	GO:0009805	Coumarin biosynthetic process	2.76×10^{-06}
	GO:0009809	Lignin biosynthetic process	2.67×10^{-05}
	GO:0010167	Response to nitrate	3.00×10^{-05}
	GO:0009835	Fruit ripening	5.71×10^{-05}
	GO:0006032	Chitin catabolic process	2.03×10^{-04}
	GO:0000024	Maltose biosynthetic process	3.56×10^{-04}
	GO:0009409	Response to cold	3.80×10^{-04}
	GO:0015706	Nitrate transport	6.14×10^{-04}
	GO:0006561	Proline biosynthetic process	8.06×10^{-04}
	GO:0005983	Starch catabolic process	1.18×10^{-03}
	GO:0042744	Hydrogen peroxide catabolic process	1.27×10^{-03}
	GO:0042432	Indole biosynthetic process	1.67×10^{-03}
	GO:0080009	mRNA methylation	1.87×10^{-03}
	GO:0055114	Oxidation reduction process	2.06×10^{-03}
	GO:0016998	Cell wall macromolecule catabolic process	2.38×10^{-03}
	GO:0009821	Alkaloid biosynthetic process	2.98×10^{-03}
	GO:0006568	Tryptophan metabolic process	3.83×10^{-03}
	GO:0018283	Iron incorporation into metallo-sulphur cluster	3.83×10^{-03}
	GO:0006537	Glutamate biosynthetic process	4.01×10^{-03}
	GO:0019079	Viral genome replication	5.53×10^{-03}
	GO:0005987	Sucrose catabolic process	6.73×10^{-03}
	GO:0019253	Reductive pentose-phosphate cycle	7.78×10^{-03}
	GO:0000051	Urea cycle intermediate metabolic process	7.78×10^{-03}
	GO:0006080	Substituted mannan metabolic process	9.41×10^{-03}
	GO:0019756	Cyanogenic glycoside biosynthetic process	9.41×10^{-03}
	GO:0031408	Oxylipin biosynthetic process	1.02×10^{-02}
	GO:0009753	Response to jasmonic acid stimulus	1.62×10^{-02}
	GO:0009853	Photorespiration	1.80×10^{-02}
	GO:0031640	Killing of cells of other organism	2.04×10^{-02}
	GO:0009834	Secondary cell wall biogenesis	2.21×10^{-02}
	GO:0015947	Methane metabolic process	2.48×10^{-02}
	GO:0080168	Abscisic acid transport	2.56×10^{-02}
	GO:0006821	Chloride transport	3.43×10^{-02}
Up-regulated	GO:0009408	Response to heat	3.91×10^{-57}
	GO:0000022	Mitotic spindle elongation	8.77×10^{-32}
	GO:0042254	Ribosome biogenesis	1.52×10^{-22}
	GO:0042542	Response to hydrogen peroxide	2.59×10^{-22}
	GO:0009644	Response to high light intensity	2.81×10^{-21}
	GO:0042026	Protein refolding	7.60×10^{-11}
	GO:0043335	Protein unfolding	4.33×10^{-07}
	GO:0009816	Defence response to bacterium, incompatible interaction	5.68×10^{-06}
	GO:0046685	Response to arsenic-containing substance	1.39×10^{-04}
	GO:0006012	Galactose metabolic process	2.62×10^{-03}
	GO:0072488	Ammonium transmembrane transport	3.19×10^{-03}
	GO:0035071	Salivary gland cell autophagic cell death	4.10×10^{-03}
	GO:0010021	Amylopectin biosynthetic process	5.85×10^{-03}
	GO:0051131	Chaperone-mediated protein complex assembly	5.85×10^{-03}
	GO:0060179	Male mating behaviour	5.85×10^{-03}
	GO:0042775	Mitochondrial ATP synthesis coupled electron transport	1.07×10^{-02}
	GO:0007444	Imaginal disc development	1.08×10^{-02}
	GO:0019852	L-ascorbic acid metabolic process	1.44×10^{-02}
	GO:0008340	Determination of adult lifespan	1.69×10^{-02}
	GO:0006624	Vacuolar protein processing	2.00×10^{-02}
	GO:0048066	Developmental pigmentation	2.38×10^{-02}
	GO:0007411	Axon guidance	3.11×10^{-02}
	GO:0008407	Chaeta morphogenesis	3.56×10^{-02}
	GO:0035966	Response to topologically incorrect protein	3.59×10^{-02}
	GO:0000915	Cytokinesis, actomyosin contractile ring assembly	3.94×10^{-02}
	GO:0051270	Regulation of cellular component movement	3.94×10^{-02}
	GO:0010998	Regulation of translational initiation by eIF2 alpha phosphorylation	3.94×10^{-02}
	GO:0097285	Cell type-specific apoptotic process	3.99×10^{-02}

FDR, false discovery rate-corrected *P*-value; GO, gene ontology.

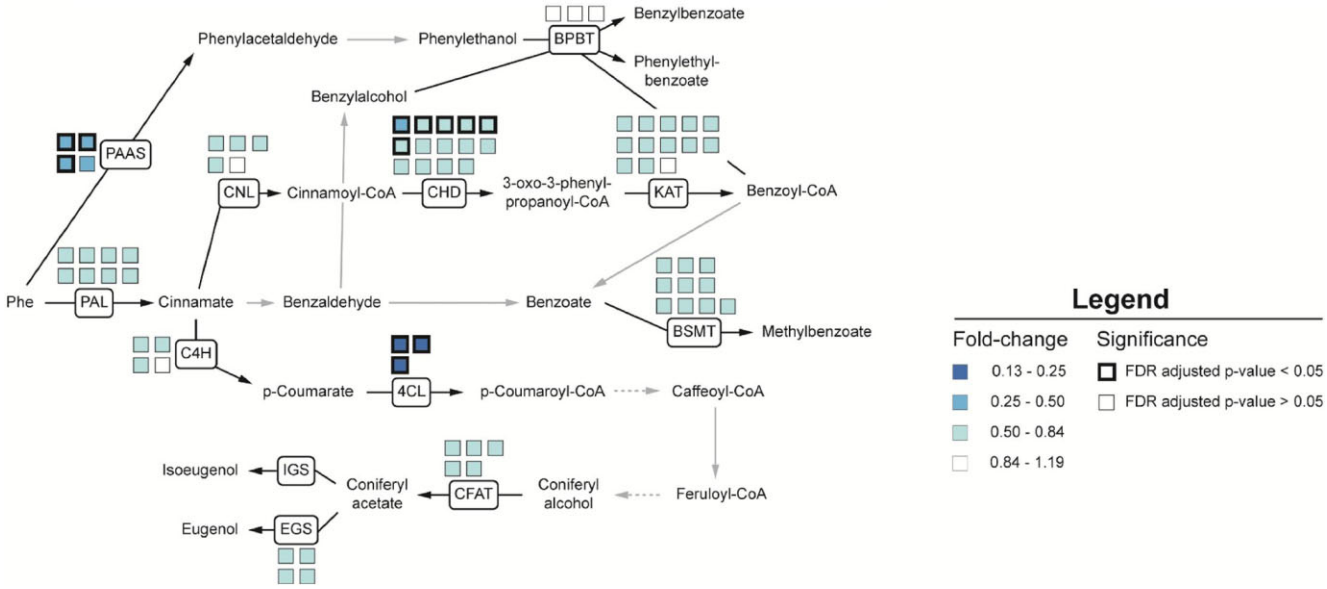

Figure 7. Changes in expression of volatile biosynthetic genes and transcription factors in *35S:PAP1* transgenic flowers grown at 22 versus 28 °C. Each box represents a contig annotated to genes involved in the benzenoid/phenylpropanoid network. Blue-coloured boxes signify a decrease in gene expression at 28 °C as compared with 22 °C. Significant changes in gene expression (FDR-adjusted *P*-value < 0.05) are illustrated by a heavy outline around the box. Abbreviations: 4CL, 4-coumarate:CoA ligase; BPBT, benzoyl-CoA:benzyl alcohol/2-phenylethanol benzoyltransferase; BSMT, S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase; C4H, cinnamate 4-hydroxylase; CFAT, coniferyl alcohol acetyltransferase; CHD, cinnamoyl-CoA hydratase-dehydrogenase; CNL, cinnamate:CoA ligase; EGS, eugenol synthase; IGS, *iso*eugenol synthase; KAT, 3-ketoacyl-CoA thiolase; PAAS, phenylacetaldehyde synthase; PAL, phenylalanine ammonia lyase; Phe, phenylalanine.

examined the levels of volatiles accumulated in and emitted by flowers of *Petunia axillaris* grown under continuous day/night ambient temperatures of 20, 25, 30 and 35 °C with 12/12 h (light/dark) photoperiod. Similar to the results presented here, the level of volatile phenylpropanoids in the internal pool decreased as the ambient temperature rose, leading the authors to suggest the involvement of metabolism in the temperature effect on scent. Those authors also analysed the levels of emitted volatiles at a single point during flower development, at 2300–2400 h 1 dpa, using solid phase microextraction (SPME)–GC–MS. Emission of volatiles from flowers at this time point decreased in plants grown at 30 versus 35 °C; at temperatures between 20 and 30 °C, volatile emissions increased. The latter increase observed by Sagae *et al*. (2008) contrasts with the results presented here, perhaps due to the different genetic backgrounds of the analysed petunia species (which may have different high-temperature-sensing thresholds) as well as to differences in the experimental setup [continuous ambient temperature versus fluctuating day/night temperature, 12/12 versus 16/8 h (light/dark) photoperiod, single-point SPME sampling]. In floral volatile analyses, the SPME sampling method is generally regarded as a more qualitative than quantitative approach to headspace analyses for several reasons: it is limited by variations in fibre-volatile affinity, it necessitates prolonged periods of equilibrium that prevent multiple simultaneous samplings, and the absorption of volatiles by the fibre depends on environmental conditions (including temperature and humidity). Although

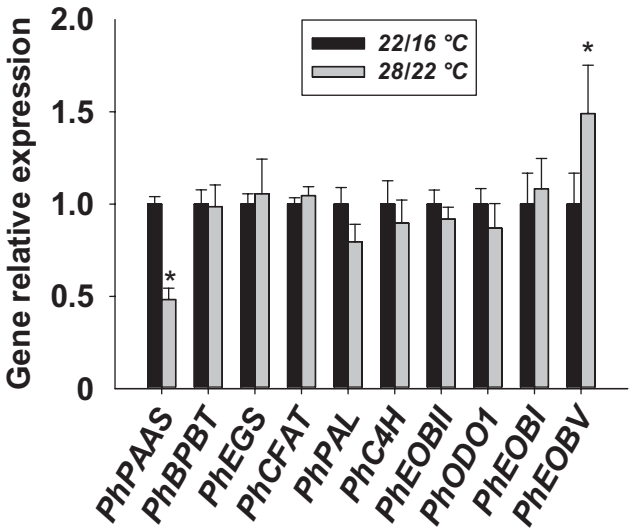

Figure 8. Scent-related gene expression in *35S:PAP1* transgenic flowers grown at either 22/16 or 28/22 °C day/night. Quantitative real-time PCR analysis was performed on *PhPAAS*, *PhBPBT*, *PhEGS*, *PhCFAT*, *PhPAL*, *PhC4H*, *PhEOBII*, *PhODO1*, *PhEOBI* and *PhEOBV* transcripts in corollas (1 dpa at 1300 h) of *35S:PAP1*-expressing 'Blue Spark' petunia plants. Presented data were normalized to that of 22/16 °C day/night, with SEs indicated by vertical lines. Significance of differences between treatments (*$P \leq 0.05$, $n = 3–5$) was calculated using Student's *t*-test based on the raw transcript level data normalized to *PhActin*.

quantification by SPME may be less useful when applied to the floral headspace, it can be utilized as a relatively quantitative approach in analyses of less complex compound mixtures if the sampling parameters are meticulously controlled and suitable standards are used (Tholl *et al*. 2006).

In addition to the negative effect of heat on floral scent production in *Petunia* × *hybrida*, increased ambient temperature resulted in a modified composition of the scent bouquet. This was a result of the differential effects of temperature on scent production, in which compounds were affected by temperature to different degrees (Figs 1–3). This effect could be due to either a differential response of the branches of the phenylpropanoid pathway to environmental stimuli or to individual compounds' physicochemical attributes. Interestingly, the effect of temperature increase on the level of accumulated volatile compounds was stronger in the morning when scent emission is at its lowest (Zvi *et al*. 2008; Spitzer-Rimon *et al*. 2010). This difference in the volatile bouquet trapped in petal cells at different time points reveals an additional level of complexity in the effect of ambient temperature on scent production. It would be of great interest to evaluate the effect of modified scent bouquet composition on the mutual relationship between plants and their respective pollinators in the field.

The elevated ambient temperature led to an overall strong reduction in most scent compounds originating from the phenylpropanoid pathway. It was therefore plausible to assume that temperature might have an effect on the expression of genes involved in phenylpropanoid biosynthesis. Indeed, a comprehensive analysis of the expression of scent-related genes under different temperature regimes showed that the decreased level of scent compounds could be attributed to that of the corresponding genes' transcript accumulation (Figs 4 & 5). A consistent and direct relationship was found between the levels of genes encoding end product enzymes and those of corresponding emitted or accumulated volatiles. For example, in line P720, a high-temperature-induced decrease in eugenol emission/internal pool levels was accompanied by a reduction in *PhEGS* transcript, while emitted/accumulated levels of *iso*eugenol remained the same, similar to those of *PhIGS* mRNA expression. It should be noted, however, that in addition to some genotype-specific responses to temperature (i.e. levels of *PhBPBT* and *PhCFAT* transcripts in P720 versus Blue Spark), here as in other studies (Orlova *et al*. 2006; Maeda *et al*. 2010; Song & Wang 2011), the decreases in volatile and transcript levels were not always to the same extent (i.e. a steeper reduction in volatile compared with transcript level or vice versa). This can be attributed to the fact that the level of final products is not exclusively determined by the expression level or even activity of the end product enzymes; other elements are involved such as substrate availability and metabolic flux within the pathway (Blount *et al*. 2000; Zvi *et al*. 2008; Alam & Abdin 2011; Farhi *et al*. 2011). Indeed, the expression of several gene-encoding enzymes that are active at the different points/branches of the pathway (namely *PhPAL*, *Ph4CL*, *PhCNL*, *PhKAT* and *PhC4H*), as well as enzymes in the shikimate pathway (*PhEPSPS*, *PhADT-1*, *PhDAHPS*, *PhCS*, *PhPPA-AT* and *PhCM*), were also strongly down-regulated by elevated temperatures (Figs 4 & 5). Therefore, the changes in scent production in *Petunia* × *hybrida* flowers at elevated temperatures were a result not only of transcript levels of enzymes catalysing the final steps of the reactions but also of the expression level of genes involved in substrate availability and maintenance of metabolic flux.

Recent data presented in several studies (reviewed by Colquhoun & Clark 2011) emphasize the crucial role of transcription factors in regulating volatile phenylpropanoid levels. These factors determine the level of volatiles through direct activation of a number of scent-related genes in the pathway (Verdonk *et al*. 2005; Spitzer-Rimon *et al*. 2010, 2012). Because most of the scent-related genes were found to be down-regulated as a result of high-temperature growth conditions, it was hypothesized that *PhEOBI*, *PhEOBII* and/or *PhODO1* would be subjected to temperature-driven down-regulation. However, elevated temperature treatment did not affect the transcript levels of these genes, whereas the expression level of the *PhEOBV* transcript, a wide-range transcriptional repressor of the pathway (Spitzer-Rimon *et al*. 2012), increased at high temperature. Overall, these data raise the possibility that the decrease in transcript levels of scent-related genes and the consequent reduction in floral scent under high-temperature conditions is due to increased levels of negative transcriptional regulators, for example, *PhEOBV*, rather than decreased levels of the positive regulators, for example, *PhEOBI*, *PhEOBII* and *PhODO1*. It should be noted that other mechanisms, such as the translational/post-translational machinery or response to temperature of as yet unidentified MYB-interacting proteins, may account for decreased activity of EOBI, EOBII and ODORANT1. A somewhat similar trend of increased transcripts of phenylpropanoid–pathway repressors was reported in *Arabidopsis thaliana* plants grown for 7 d at high temperatures (Rowan *et al*. 2009). Expression analysis of the phenylpropanoid pathway's negative regulators *AtMYB3*, *AtMYBL2* and *AtMYB6* revealed up-regulated levels of these transcripts in response to high-temperature conditions. Moreover, the expression of two MYB transcriptional activators of the phenylpropanoid pathway, *AtPAP1* and *AtPAP2* (Borevitz *et al*. 2000), was not affected by this treatment.

Although floral scent and colour production do not coincide developmentally, as colour accumulation precedes that of scent production (Jackson *et al*. 1992; Dudareva *et al*. 2000; Colquhoun *et al*. 2010b), the observed similar down-regulation of both (Figs 1 & 3) may not be surprising as the two groups of compounds share common substrates that are metabolized by the same gene products (for instance PAL, 4CL and C4H). A negative effect of temperature on anthocyanin content is well documented in many horticultural plants. For example, in flowers of petunia hybrid V23 × R51, high-temperature (32/27 °C day/night) conditions reduced anthocyanin content, together with inhibition of *PhCHS* (chalcone synthase) gene expression (Shvarts *et al*. 1997). This effect was also evident in aster hybrids (*Aster ericoides* × *Aster pilosus*) (Shaked-Sachray *et al*. 2002), where the reduction in

pigment levels at high temperatures (29/21 °C day/night) compared with 23/15 °C day/night was partially attributed to a decrease in *PAL* expression. The coordinated down-regulation of showy traits such as scent and colour under high-temperature conditions reaffirms the link between these functionally complementary metabolic branches.

Ectopic expression of *AtPAP1* in several plant systems leads to increased production of phenylpropanoids (both pigments and volatiles) by inducing the expression of a number of phenylpropanoid pathway genes (Zhou *et al*. 2008; Zvi *et al*. 2012; Qiu *et al*. 2013). Furthermore, although petunia Blue Spark flowers do not emit volatiles during the day, ectopic expression of *AtPAP1* overcomes this diurnal pattern of scent production, leading to volatile emission at essentially the same level during the day and at night (Zvi *et al*. 2008). With that in mind, we tested whether the adverse effect of increased temperature on scent production could be negated in flowers with enhanced transcriptional activation of the phenylpropanoid pathway genes by *AtPAP1*. Indeed, the levels of volatiles generated by flowers of *PAP1* expressing Blue Spark plants grown at high temperature were essentially the same or higher than those of the transgenic plants grown under standard conditions (22/16 °C day/night) (Fig. 6). This is in contrast to non-transgenic flowers, in which pools decreased by ~8–50% under high versus standard growth conditions (see Fig. 3). Moreover, levels of most relevant transcripts in *35S:PAP1* transgenic flowers (based on RNA-Seq and real-time qPCR) were little or not affected by high temperature as compared with non-transgenic control flowers similar to those of volatiles. The only exception among genes directly involved in scent production was *PhPAAS*, whose transcript level was reduced at high temperature; nevertheless, the level of phenylacetaldehyde, the product of PAAS enzymatic activity, was not affected by the elevated temperature regime. Overall, it seems that expression of *PAP1* can reverse the negative effect of temperature via transcriptional up-regulation of the pathway; however, transcriptional regulation is only one element of the complex multidimensional sensing machinery responding to ambient temperature. For example, in contrast to the reduction in volatile production upon long-term exposure of plants to high temperature, levels of emitted volatiles were enhanced following brief exposure of the flowers to high temperature. This might be explained by a transient increase in vapour pressure as suggested by Sagae *et al*. (2008).

The detailed molecular mechanisms by which plants perceive and mediate temperature cues are not entirely clear. Recent studies have suggested several hypotheses regarding the identity of the sensing component, including changes in membrane fluidity, ion channel status, protein stability, epigenetic modifications and changes in enzymatic kinetics (McClung & Davis 2010; Mittler *et al*. 2011; Proveniers & van Zanten 2012). Further research is therefore necessary to elucidate which of these proposed sensing components are involved in mediating the temperature-signal transduction in the context of volatile phenylpropanoid production.

The frequency of extreme and unseasonable heat episodes is expected to increase, along with mean global and local temperatures. Climatic projections predict a strong rise of between 1.1 and 4.8 °C in global surface temperature by the end of the century relative to the period 1986–2005 (Stocker *et al*. 2013). This trend might weaken plant–pollinator mutualism not only by increasing phenological decoupling between the organisms but also due to its negative effect, described herein, on phenylpropanoid-based floral scent. In contrast to phenylpropanoid-derived floral scent, environmental effects on the production of terpenoid plant volatiles have been studied in depth. It has been shown that over the last 30 years, the changes in heat, drought and CO_2 levels have caused an increase of ~10% in biogenic volatile organic compound (BVOC) emission from plants, with terpenoids representing the bulk share of BVOCs (Guenther *et al*. 1995; Fall & Wildermuth 1998; Penuelas & Staudt 2009; Loreto & Schnitzler 2010). This enhanced production is typically connected to their proposed role in reducing the oxidative stress and damage to membranes caused by high temperatures (Loreto *et al*. 2006). Hence, a multifaceted effect of increasing ambient temperature on plant–pollinator interactions can be expected.

Applying a transcriptional up-regulation approach might be useful in overcoming the long-term negative effect of temperature on floral scent production in both floricultural (where scent and colour are the main traits dictating consumer preference) and pollination-dependent crops. Indeed, it has been recently shown that bees can discriminate between volatiles emitted by *35S:PAP1* transgenic and non-transgenic control rose flowers producing a rich floral bouquet composed of terpene- and phenylpropanoid-derived volatiles (Zvi *et al*. 2012). A detailed understanding of the transcriptional machinery as well as of post-transcriptional mechanisms involved in sensing and transmitting temperature cues should allow, in the future, fine optimization of floral scent production under various adverse ambient conditions.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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Figure S1. Short-term (8 h) exposure of petunia cv. Blue Spark flowers (at 1 dpa) to high-temperature conditions increases the level of benzaldehyde emission. 'Blue Spark' plants were grown for 1 month at 22/16 °C and dynamic headspace analysis of benzaldehyde was initiated following their transfer (at 1600 h) to 28/22 °C conditions. For comparison, benzaldehyde levels were also analysed in flowers of plants grown only under 22/16 and 28/22 °C temperature conditions. Dynamic headspace analyses of benzaldehyde were assayed using GC–MS. Values are the averages of four to seven independent experiments with standard errors indicated by vertical lines. The significance of the differences in emission levels between temperature regimes was calculated using Tukey's all pairwise multiple comparison procedure following one-way analysis of variance (ANOVA). Values with different letters are significantly different at $P < 0.05$.
Table S1. Sequences of primers used in quantitative real-time PCR analyses of petunia cv. Blue Spark and line P720.