Regulation of cucumber (*Cucumis sativus*) induced defence against the two-spotted spider mite (*Tetranychus urticae*)

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To my beloved wife, Xiaohua

惟真理值得追寻

惟真爱值得跟随

Contents

Chapter 1

General introduction

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Insect defence in plants

Pests have commonly been a threat to agriculture since people started using plants as crops 10,000 years ago because insects, mites and other herbivores feed on crops and decrease the yield and quality of products (Oerke, 2006). At least as early as 2500 B.C., ancient Sumerians started to use sulphur compounds for insect control in their crops (Pedigo and Rice, 2009) and the earliest written example of biological control of insects dates back to 304 A.D. describing the use of ants by Chinese farmers to prevent citrus fruit tree damage by insects such as *Rhynchocoris humeralis* and *Anoplophora chinensis* (Han, 304, Huang and Yang, 1987).

Nowadays various types of pesticides are widely used to control pests and they contribute largely to reducing crop losses in modern agriculture (Oerke, 2006). However, many of these pesticides are toxic and may cause harm to the health of people (Bassil et al., 2007, Sanborn et al., 2007), pollute the environment including air, soil and water (Kole et al., 2001, Sokolovski and Dombalov, 2007, Piel et al., 2012), and reduce biodiversity (Geiger et al., 2010). To produce agricultural products in a safer way, host plant resistance and biological control, which uses natural enemies to control pests, are attractive alternatives for plant protection. To better exploit these approaches, we need to improve our understanding about the underlying mechanisms. Because of their co-existence with herbivores, plants have evolved mechanisms to protect themselves from the threat of being consumed. In general, plants defences can either directly affect the herbivores or indirectly through attraction of the natural enemies of the herbivores (Dicke and Sabelis, 1988). Plants defences can be either constitutive or inducible (Fig. 1). Constitutive defence mechanisms include physical barriers (e.g. plant hairs, latex and leaf toughness) or defensive chemicals (e.g. toxic compounds) which can repel or kill herbivores (Duffey and Stout, 1996, Hanley et al., 2007, Wisdom, 1985). Inducible defence only occurs or is enhanced upon the attack of herbivores (Mithofer and Boland, 2012). The inducible defence involves a series of reactions that happen in the plant at different levels including to sense the attack, followed by signal transduction, transcriptional regulation, translation, and reconfiguration of metabolism. In this thesis, I focus on inducible plant defence, especially inducible indirect defence and I study that in a well-established model for indirect defence research, the tritrophic interaction between cucumber, two-spotted spider mites and predatory mites (Dicke et al., 1990b, Mercke et al., 2004, Kappers et al., 2010, Kappers et al., 2011).

Cucumber and spider mites

In my thesis I use cucumber (*Cucumis sativus*) and two-spotted spider mites (*Tetranychus urticae* Koch) as study system. Most likely domesticated in India more than 3000 years ago (Sebastian et al., 2010), cucumber is nowadays grown world-wide. It is among the four most

consumed vegetables with onion, tomato and cabbage. In 2013 the annual world gross production value of cucumber reached around 35 billion US dollar (http://faostat3.fao.org/home/E, final 2013 data). In addition to its agricultural importance, cucumber has a number of advantages to be used as a model plant for scientific research. Its genome and the number of putative genes is relatively small (Huang et al., 2009) and it has a relatively short life cycle. Moreover, cucumber employs a number of interesting defence mechanisms against herbivores. For example, the bitter compound cucurbitacin C (Fig. 1) present in cucumber foliage and fruit discourages multiple herbivores (Metcalf and Lampman, 1989, Metcalf et al., 1980, Tallamy and Krischik, 1989, Tallamy et al., 1997, Agrawal et al., 2002, Balkema-Boomstra et al., 2003) although specialist herbivores, such as spotted and striped cucumber beetles, specialize on cucurbitacin containing cucumber plants and in this way avoid their natural enemies (Da Costa and Jones, 1971, Haynes and Jones, 1975, Ferguson and Metcalf, 1985). Genetic, genomic and transcriptomic resources for cucumber have increased rapidly (Huang et al., 2009, Ren et al., 2009, Guo et al., 2010, Li et al., 2011, Lv et al., 2012, Qi et al., 2013) and facilitate to explore the mechanisms underlying various biological processes in cucumber, including defence mechanisms, easier than before.

Spider mites, next to thrips, whiteflies and nematodes are the primary pests that harm greenhouse cucumber production. Spider mites are important agricultural pests for many crops. They pierce the epidermis and feed on the contents of mesophyll cells, resulting in chlorosis, a decrease of total chlorophyll content and finally loss of photosynthetic capacity of the leaves (Park and Lee, 2002). Spider mites can infest more than one thousand different plant species of over one hundred families (Van Leeuwen et al., 2010). Moreover, spider mites can develop rapidly, and under suitable conditions finish their life cycle within eight days (Jeppson. et al., 1975). Together with a high fecundity and haplo-diploid sex determination, spider mites are able to develop rapid resistance to pesticides (Van Leeuwen et al., 2010). Biological control is an attractive alternative approach to protect crops from the threat of spider mites. For example, the predatory mite *Phytoseiulus persimilis* is widely used for biological control of the two spotted spider mite *Tetranychus urticae* in greenhouse crops. This predator is attracted to volatiles emitted by lima bean (Dicke et al., 1999) and cucumber (Takabayashi et al., 1994, Kappers et al., 2011) plants infested by the two-spotted spider mite. *Neoseiulus californicus* can also effectively control spider mites (McMurtry and Croft, 1997). These two natural enemies of spider mites are commercially available and are commonly used in greenhouses or gardens in the US and Europe to control two-spotted spider mites.

Using predatory mites to control spider mites requires that the predator can locate its prey. Being blind and deaf, predatory mites rely on odours to find their prey (Fig. 1). While many herbivores have evolutionarily adapted to be silent in terms of odours, plants release volatile blends upon herbivore infestation that quantitatively and qualitatively differ from the blend emitted by non-infested plants. Cucumber plants emits a blend of induced volatiles

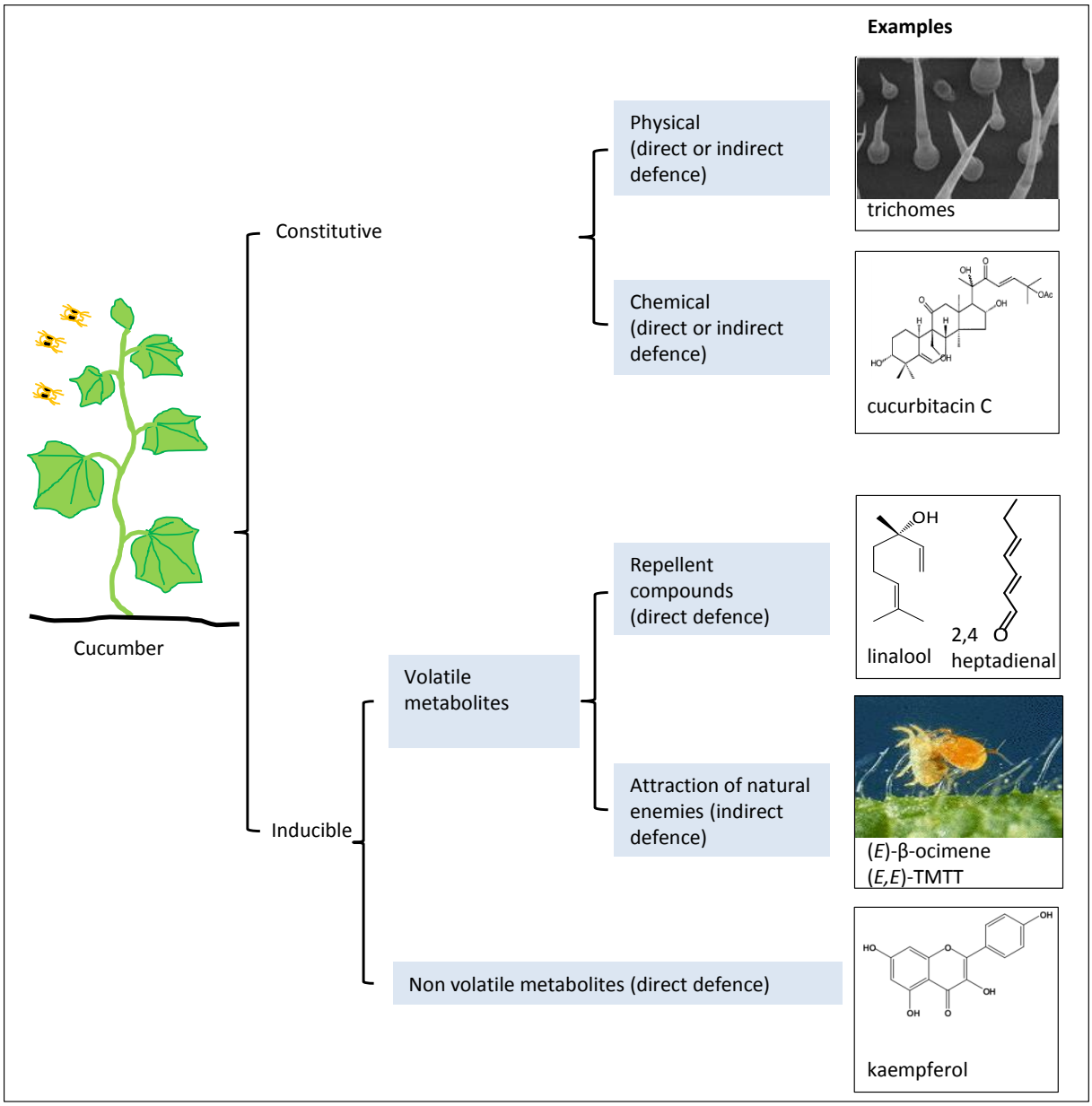

Fig. 1. Classification of defences to herbivores, using cucumber, spider mites and predatory mites as model. Illustrating examples: cucumber trichomes hindering herbivores (photo from Zhao et al., 2015), triterpenoid cucurbitacin C, chemically repellent to spider mites (Agrawal et al. 2002; Balkema-Boomstra et al. 2003); spider-mite induced 2,4 heptadienal and linalool repellent to spider mites (Kappers et al., 2010, Nyalala et al., 2013, www.google.com/patents/US4933371); (*E*)-β-ocimene and (*E*)-TMTT [(*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene] induced upon spider-mite herbivory and attractive to predatory mites [(van Wijk et al., 2008, Kappers et al., 2011); photo credits: Koppert Biological Systems BV]; flavonoid phytoalexins like kaempferol induced upon two-spotted spider-mite infestation (unpublished results, I.F. Kappers and R. de Vos).

containing over 24 different compounds upon infestation by two-spotted spider mites (Kappers et al., 2010). The predominant compounds among these volatiles are terpenoids including (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E,E*)-α-farnesene, and (*E*)-β-ocimene (Takabayashi et al., 1994, Kappers et al., 2010). Moreover, variation in the quantity and quality of spider-mite induced volatiles was found between different cucumber varieties with

the quality of the blend of volatiles being more important for attraction of *P. persimilis* than the quantity (Kappers et al., 2011). The amount of (*E*)-β-ocimene, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and two other unidentified compounds positively correlated with attractiveness (Kappers et al., 2011). The diversity of the emitted HIPV blends among the cucumber germplasm and the correlation of certain compounds to attractiveness to natural enemies imply that crops with specific profiles of volatiles and enhanced indirect defence could be developed via breeding (Dicke et al., 1990a, Kappers et al., 2011, Stenberg et al., 2015).

Profiling transcriptome changes of plants in response to herbivory is a good approach to start unravelling the mechanisms of inducible plant defence. However, to date only few papers on cucumber transcriptomics have been reported. The first one being a custom-made microarray containing 713 cDNA fragments of cucumber and used to study transcriptional changes in cucumber leaves in response to jasmonic acid (JA), mechanical damage and infestation by two-spotted spider mites (Mercke et al., 2004). The transcripts of several genes including the ones encoding an (*E*)-β-ocimene/(*E,E*)-α-farnesene synthase and a lipoxygenase in cucumber were found to be induced by two-spotted spider mites and the increased expression of these genes correlated with the enhanced emission of terpenoids and green leaf volatiles upon herbivory (Mercke et al., 2004). Furthermore, the blend of volatiles induced by JA application was very similar to the blend induced by spider mites, which implies involvement of the JA pathway in the transcriptional regulation of the associated genes. More recently RNA-seq was used to reveal gene expression profiles in different cucumber organs (Li et al., 2011) and gene expression changes in cucumber leaves in response to *Pseudoperonospora cubensis* downy mildew (Adhikari et al., 2012). In this thesis, I use RNA-seq to analyse global changes in gene expression in cucumber in response to a chelicerate cell content feeding herbivore, the two-spotted spider mite *T. urticae*.

Detection of invasion and early signalling.

Feeding by herbivores on plants impacts the plant both physically and chemically and these impacts are recognized separately. The physical damage that is inflicted depends on the feeding style of the herbivore. Cell-content feeders like spider mites and thrips make moderate physical damage per individual, while chewing herbivores (e.g. caterpillars) cause significant physical damages as they bite off and ingest large parts of tissues (Ehlting et al., 2008). In contrast, phloem-feeding herbivores such as aphids suck the phloem sap and hence inflict little mechanical damage (Ehlting et al., 2008). In addition to physical damages, plants can also perceive chemical signals (e.g. effectors or elicitors) from the body, eggs or oral secretion of the herbivore (Alborn et al., 1997, Heil, 2009, Mori and Yoshinaga, 2011, Bonaventure et al., 2011). Together, these physical and chemical stimuli from herbivores trigger early signalling responses such as a Ca^{2+} flux and reactive oxygen species burst [Fig. 2,

Wu and Baldwin (2010)]. The early recognition of different feeding styles and chemical signals from herbivores subsequently activates distinct signalling processes involving different phytohormones (Fig. 2). JA, salicylic acid (SA) and ethylene were shown to be the three major phytohormones that mediate the regulation of plant defence (Wu and Baldwin, 2010). The JA/ethylene signalling pathway is normally induced by wounding and tissue-damaging herbivores such as caterpillars (McCloud and Baldwin, 1997), while the SA pathway is primarily activated by phloem-feeders such as aphids (Moran and Thompson, 2001). Both JA and SA seem to be involved in the response of plants to infestation by piercing and sucking herbivores like spider mites and thrips (Dicke et al., 1999, Ament et al., 2004, Abe et al., 2009, Zhurov et al., 2014), although in principle they were reported to act antagonistically to each other (Maffei et al., 2007). Changes in the content of phytohormones in a plant that is infested by herbivores subsequently results in the release or inhibition of the activities of transcription factors (TFs, Fig. 2). For example, in the JA signalling pathway, JA-Ile, the conjugation product of JA and iso-leucine which is formed by JAR (Staswick and Tiryaki, 2004), binds to COI1 (CORONATINE INSENSITIVE 1) and facilitates the physical interaction with and degradation of JAZs [JASMONATE ZIM-DOMAIN PROTEINS, (Chini et al., 2007, Thines et al., 2007, Yan et al., 2009)]. Under unchallenged conditions, JAZs bind to JA-responsive TFs such as MYC2 and inhibit gene transcription. Upon herbivory, the increase of JA-Ile results in decreased levels of the JAZs, thereby activating JA-responsive TFs and subsequent gene transcription.

TFs are proteins that are able to recognize and bind to DNA at specific regulatory sequences (Latchman, 1997, Wittkopp and Kalay, 2012) and hence either positively or negatively regulate the expression of relevant genes (Karin, 1990). More than sixty TF families have been identified in different plant species and classified according to conserved domains of the proteins (Perez-Rodriguez et al., 2010). Multiple TF families are reported to play a role in transcriptional regulation in plant defence, especially MYB, bHLH, WRKY, AP2/ERF, NAC and bZIP TFs (Seo and Choi, 2015). For instance, AtMYB51 and AtMYB75 in Arabidopsis, PpMYB134 in *Populus tremuloide* and NaMYB8 in *Nicotiana attenuata* have been reported to be essential for the biosynthesis of various anti-herbivore secondary metabolites (Gigolashvili et al., 2007, Borevitz et al., 2000, Mellway et al., 2009, Gális et al., 2006, Kaur et al., 2010). MYC TFs which contain the bHLH domain are important for JA signalling pathways as described above. Multiple WRKY, ERF, NAC and bZIP TFs are also found to be important for plant defence (Skibbe et al., 2008, Moffat et al., 2012, Zhao et al., 2012, Seo and Choi, 2015). The DNA motifs bound by TFs are generally called *cis*-acting regulatory elements (CAREs) and are usually present in the sequence adjacent to the target gene or within the gene (Wittkopp and Kalay, 2012). Deferent classes of TFs prefer different binding motifs, for example, bHLHs can recognize the E-box (Toledo-Ortiz et al., 2003), ERFs bind to GCC boxes- (Zarei et al., 2011) and WRKY TFs bind to the W-box (Sun et al., 2003).

By binding to these target sites TFs can stabilize or block the binding of RNA polymerase to the DNA (Gill, 2001), catalyse the acetylation or deacetylation of histone proteins (Narlikar et

al., 2002), and recruit co-activator or co-repressor proteins to form transcription factor DNA complexes (Xu et al., 1999). All these processes can result in different expression of target genes, either activation or repression (Karin, 1990). Changes in TFs thus result in changes in the expression of genes in the plant, for example after an impact such as herbivory. These gene expression changes can be monitored, for example using micro-array analysis or RNA-sequencing (RNA-seq). Many studies used microarrays to analyse transcriptional changes in various plant species during infestation by herbivores. For example, in Arabidopsis gene-expression changes were profiled in response to caterpillars, thrips, aphids and spider mites (Reymond et al., 2004, De Vos et al., 2005, Little et al., 2007, Kusnierczyk et al., 2007, Zhurov et al., 2014). Among these studies, from about one hundred to around two thousand genes were identified as differentially expressed genes (DEGs), that is genes of which the expression was up- or down-regulated in response to herbivory. In rice, microarray analysis identified almost 200 genes significantly up-regulated when plants were damaged by fall armyworm (Yuan et al., 2008). Tomato transcriptome responses to spider mites were characterized with a custom-made micro-array containing expressed sequence tags (Kant et al., 2004) or a commercial whole-genome exon array (Martel et al., 2015). However, micro-array technology is restricted by the available DNA probes on the array and the sensitivity of the hybridization to extremely low or high transcript copy numbers. RNA-seq does not have these disadvantages and therefore could give a more comprehensive insight into novel, rare or low-abundance transcripts with a higher specificity and sensitivity (Zhao et al., 2014). A number of studies used RNA-seq to track pathogen-induced transcriptional changes, for example in Arabidopsis (Zhu et al., 2013), rice (Kawahara et al., 2012), soybean (Kim et al., 2011), cotton (Xu et al., 2011) and banana (Li et al., 2012) identifying hundreds to thousands of DEGs. However, to date only a few studies used RNA-seq to analyse transcriptional changes in response to herbivory. For example, transcriptome changes in *N. attenuata* after elicitation by FACs (fatty acid-amino acid conjugates), major components in the oral secretion of *Manduca sexta,* were analysed using 454 sequencing which resulted in the identification of genes mediating early responses to this insect (Gilardoni et al., 2010).

Finally, as a consequence of changes in the expression of genes involved in metabolism, the biosynthesis of metabolites will alter upon herbivory (Fig. 2). Plants can produce various defensive chemicals to repel or kill herbivores. For example, in tobacco the content of the extremely toxic pyridine alkaloid nicotine increases upon herbivory to repel a variety of herbivores (Steppuhn and Baldwin, 2007, Steppuhn et al., 2004). In brassicaceous species the content of glucosinolates increases upon herbivory by multiple herbivores (Hopkins et al., 2009). Well-known defensive metabolites in Cucurbitaceae species are cucurbitacins which confer a bitter taste to the fruit and foliage of the plants. As mentioned above, cucumber contains cucurbitacin C which is known to repel or decrease the performance of various pests including spider mites (Agrawal et al., 2002, Balkema-Boomstra et al., 2003). Moreover, cucurbitacin C was induced in local infested cotyledons as well as the first systemic leaf of cucumber plants infested by spider mites (Agrawal et al., 1999). In addition to changes in

endogenous metabolites that will affect herbivores in a direct manner, plants usually emit volatiles in response to herbivory, which can attract natural enemies of the herbivore. These herbivore-induced plant volatiles (HIPVs) mostly include green leaf volatiles (GLVs), methyl salicylate (MeSA) and various terpenoids (War et al., 2011, Dicke et al., 1990b). Olfactometric choice assays showed that MeSA and multiple terpenoids are important cues for the predatory mite *P. persimilis* to discriminate between infested and non-infested lima bean plants and even lima bean plants infested by its prey and plants infested by non-prey herbivores (Dicke et al., 1990b, De Boer et al., 2004).

Terpenoids, an important class of induced defence compounds.

Since terpenoids are among the essential HIPVs, terpene synthases (TPSs) and other enzymes that are involved in the biosynthesis of terpenoids are of particular interest to me (Fig. 2). The terpenoids comprise more than 40,000 compounds with different structures all built up from 5-carbon isoprene units (Aharoni et al., 2005). Many plant species synthesize specific terpenoids, likely to help the plant adapt to its unique ecological niche (Pichersky et al., 2006, Chen et al., 2011). Besides the function as volatiles attracting allies in indirect defence as described above (Pichersky and Gershenzon, 2002, Degenhardt et al., 2003, Kappers et al., 2005), terpenoids may also be involved in direct defence against pathogens or herbivores (Bohlmann et al., 2000, Balkema-Boomstra et al., 2003, Wang et al., 2004, Nagegowda, 2010).

Although there is a huge diversity of structures in the terpenoids, they are all produced from a limited number of precursors, such as geranyl diphosphate (GPP, C10) and nerolidyl diphosphate (NPP, C10) for the biosynthesis of monoterpenes, *cis*- and *trans*- farnesyl diphosphate (FPP, C15) for sesquiterpenes and geranylgeranyl diphosphate (GGPP, C20) for diterpenes (Bohlmann et al., 1998, Takahashi and Koyama, 2006, Tholl, 2006). The terpene backbone of monoterpenes, sequiterpenes and diterpenes is synthesized by TPSs while the first biosynthetic step of the triterpenoids is catalysed by oxidosqualene cyclases (Abe et al., 1993). Several conserved motifs were identified in the TPSs from different species, including the "RRX_8W", "DDXXD", "EDXXD", "NSE/DTE" and "RXR" motifs (Cao et al., 2010, Koksal et al., 2011). Among them, "RRX_8W" is located at the N-terminal and may function in cyclisation of monoterpenes. Upstream of this motif usually there is a targeting peptide in the monoterpene and diterpene synthases which facilitates the import of the protein into the plastids after which it is cleaved off in the mature protein. These targeting signals are usually absent in sesquiterpene synthases (Chen et al., 2011). The more C-terminal located conserved motif "DDXXD" is the active site for metal dependent ionisation of the substrates (Cao et al., 2010, Koksal et al., 2011). It is likely that herbivore induced terpenoids are synthesized *de novo* because genes encoding TPSs are up-regulated by herbivores in various plant species such as *Arabidopsis thaliana* (Van Poecke et al., 2001), *Oryza sativa* (Yuan et al.,

2008), *Lycopersicon esculentum* (Kant et al., 2004), *Medicago truncatula* (Arimura et al., 2008) and *Cucumis sativus* (Mercke et al., 2004). Throughout the plant kingdom, the family of *TPS* genes is a mid-sized gene family (Chen et al., 2011). In Arabidopsis, for example, about 40 putative *TPS* gene models were identified (Aubourg et al., 2002), in tomato 44 (Falara et al., 2011), and 57 in rice (Chen et al., 2011). A number of individual *TPS* genes have been isolated and encoded proteins enzymatically characterized such as a number of genes from the tomato (Falara et al., 2011) and cotton *TPS* families (Yang et al., 2013). The TPSs identified from all different plant species can be divided into 7 subfamilies (TPS-a, b, c, d, e/f, g and h) based on their putative evolutionary relationship (Chen et al., 2011). Until now, the majority of the cucumber TPSs has not been identified let alone their catalytical function determined except for the (*E*)-β-ocimene/(*E,E*)-α-farnesene synthase described above (Mercke et al., 2004). In cucumber, the backbone of the defensive triterpenoids, cucurbitacin C, is produced by the oxidosqualene cyclase, CUCURBITADIENOL SYNTHASE, encoded by the *Bi* (*BITTER*) gene, the product of which is subsequently converted by multiple P450 proteins and an acyltransferase to form cucurbitacin C (Shang et al., 2014).

Genes encoding TPSs are often localized in gene clusters in the genome. Eighteen of the 40 *TPS* genes in Arabidopsis are located in six small gene clusters (Aubourg et al., 2002). In tomato, nine *TPS* genes form a cluster and six form another one (Falara et al., 2011). The largest *TPS* gene clusters were identified in the grape genome, in which the largest cluster contains 20 complete *TPS* genes and 25 *TPS* pseudogenes (Martin et al., 2010). Phylogenetic analysis of the *TPS* families showed that most of the genes in the same cluster originated from repeated duplications and were not horizontally transferred. Often not only the TPS but also other genes involved in the biosynthesis of particular metabolites are organised in clusters, which could ensure these genes are co-inherited (Osbourn, 2010, Nutzmann and Osbourn, 2014, Chu et al., 2011, Takos and Rook, 2012), or co-regulated by chromatin modification (Wegel et al., 2009).

Visualization of changes in gene expression upon herbivory

Reporter systems enable the visualization of the expression of genes. These approaches help to elucidate the temporal and spatial changes in the expression of genes. Different reporters have been developed and GFP, GUS and LUC are the three widest used in plant research (de Ruijter et al., 2003). Generally, GFP is useful to indicate the subcellular localisation of expressed proteins, but usually auto-fluorescence of the plant material is strong and variable between plant organs making GFP unsuitable to study gene expression differences at the whole-plant level (Quaedvlieg et al., 1998). In addition, GFP needs to be observed under the microscope and can hence not be used to study dynamic changes in living samples. GUS produces stable staining, but it can only indicate tissue specificity, not dynamic and temporal changes (Jefferson et al., 1987). LUC is suitable to observe gene expression at the whole-

plant level as it can reveal real-time expression in living tissues in a sensitive and rapid way. However, the drawback of LUC is that it is hard to visualise tissue- or cell-specific expression. Moreover, the factors involved in the enzyme reaction such as oxygen, ATP and the substrate luciferin need to be carefully considered as variation in their availability may cause biased images which do not accurately correlate with gene expression (de Ruijter et al., 2003).

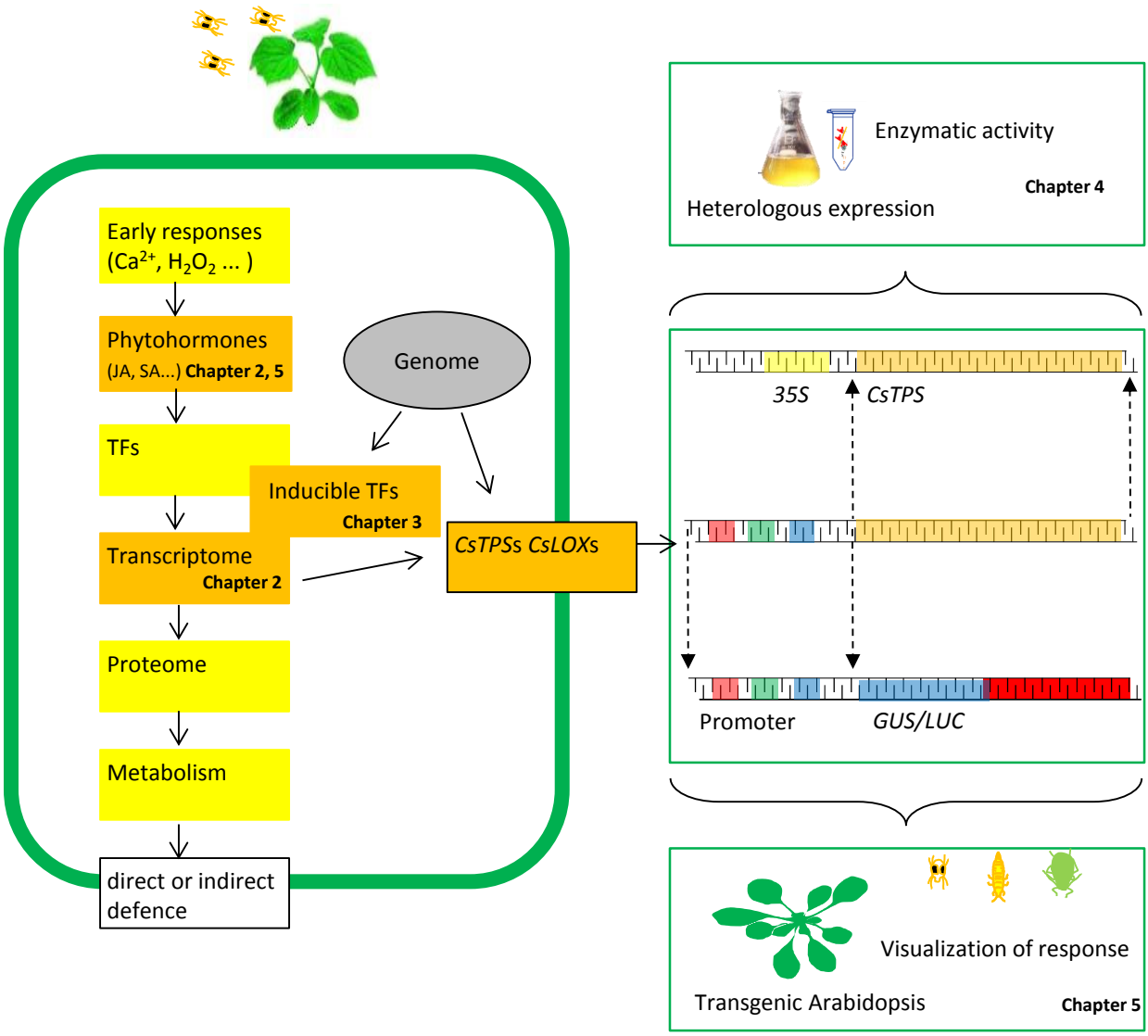

Fig. 2. Simplified scheme of cucumber responses to spider-mite infestation, including the aspects that the different chapters in this thesis address.

Scope of this thesis

In this thesis I study the underlying mechanisms of inducible defence to herbivory in cucumber, which is introduced in **this first chapter**. To achieve that goal in **Chapter 2,** I study the temporal transcriptional changes in cucumber upon herbivory by two-spotted spider mites. The goal is to identify genes responsive (positively or negatively) to feeding of spider

mites with an emphasis on genes involved in the biosynthesis and regulation of phytohormones and volatile and non-volatile metabolites. **Chapter 3** aims to understand the transcriptional regulation mechanisms involved in cucumber inducible defences using an *in-silico* analysis to identify the TFs which regulate metabolism-associated genes and the relevant CAREs on the promoter regions of these genes. The goal of **Chapter 4** is to describe the *CsTPS* gene family. I identify this gene family at the whole genome level, characterize their enzymatic function and analyse the expression of these genes upon herbivory. In **Chapter 5**, promoter activities of three selected cucumber defence related genes are studied in detail. Using different reporters, I assess whether the cucumber promoters in transgenic Arabidopsis still respond to spider mites, as well as to other biotic and abiotic factors. Finally, in **Chapter 6** I discuss the cucumber responses to spider mites, the involvement of phytohormones, transcriptional changes, defensive metabolite formation and the changes in expression of associated genes. Genes related to inducible volatile formation receive specific attention. In addition, I discuss the possible implications of my work for crop protection.

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Chapter 2

Transcriptional changes in cucumber (*Cucumis sativus*) in response to two-spotted spider-mite (*Tetranychus urticae*) infestation reveal induction and suppression of defence-related pathways

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Abstract

To obtain insight in how two-spotted spider mites (*Tetranychus urticae*) affect defence in cucumber, RNA-seq analysis was used to study early changes in the transcriptome of cucumber foliage upon herbivory by spider mites. Cucumber (*Cucumis sativus*) accessions Chinese long 9930 (with bitter foliage) and Corona (with non-bitter foliage) were infested by spider mites and gene expression analysed at different time points (one, two and three days) after infestation. Spider mites caused reconfiguration of the cucumber transcriptome from the first day of infestation onwards and up to 2348 genes were found to be differentially expressed in at least one of the two genotypes. Functional enrichment analysis of these differentially expressed genes showed that biological processes related to phenylpropanoids, L-phenylalanine and jasmonates were most strongly upregulated in response to spider-mite feeding. Also genes encoding lipoxygenases or involved in terpene biosynthesis and photosynthesis were upregulated. In contrast, genes involved in cucurbitacin C biosynthesis were downregulated. Consistent with the observed changes in the transcriptome, volatile emission and jasmonic acid content increased and cucurbitacin C content decreased upon spider-mite herbivory. This study provides a global image of the transcriptional changes in leaves of *C. sativus* in response to herbivory by *T. urticae* spider mites.

Keywords: cucumber, spider mites, RNA-seq, induced defence, lipoxygenase, terpene biosynthesis, cucurbitacin C

Introduction

During evolution, plants have armed themselves to survive in an environment full of pathogens and herbivores. The natural defence mechanisms they have evolved can be used to decrease the economic costs of pest control and to produce food in a safe and environment-friendly way. Basically, plants have two types of strategies that affect herbivores either in a direct or indirect way and both strategies can be constitutively present or be induced upon herbivory (Mithofer and Boland, 2012). Physical and chemical barriers can help to prevent herbivore attack (Duffey and Stout, 1996, Hanley et al., 2007, Wisdom, 1985). For example, plant hairs, latex and leaf toughness can hinder herbivores to access the foliage. Secondary metabolites (already present or synthesized upon attack) may also deter herbivores from feeding or reduce herbivore performance. For example, the pyridine alkaloid nicotine in tobacco species is well known to be extremely toxic to many herbivores (Steppuhn et al., 2004). Other defence-related compounds are induced or increased when plants are under attack. For instance glucosinolates in the Brassicaceae family increase when plants are attacked by herbivores and act as defensive compounds with broad negative effects on many generalists insect herbivores (Hopkins et al., 2009).

In addition to differences in the level of direct defence as a result of changes in endogenous metabolites, volatile metabolites emitted from plants in response to herbivore attack can serve as cues to attract natural enemies of the herbivores (Dicke and Sabelis, 1988). This so-called induced indirect defence depends on tri-trophic interactions between a plant, the herbivore and its natural enemy. These interactions are mediated via herbivore-induced plant volatiles (HIPVs) comprising green leaf volatiles (GLVs), ethylene, methyl salicylate, terpenoids and a number of other chemical classes (War et al., 2011). The GLVs are synthesized via the lipoxygenase pathway (D'Auria et al., 2007) while the terpenoids are synthesized by terpene synthases via the mevalonate (MVA) pathway in the cytosol or the methylerythritol 4-phosphate (MEP) pathway in the plastids (Aharoni et al., 2005).

Re-configuration of the metabolome, including endogenous compounds and emitted volatiles, is the result of prior re-configuration of the transcriptome. Plants have complex regulatory networks to control defence in which multiple phytohormones are involved that in turn activate various transcription factors and subsequently up-regulate or suppress defence-related genes (Ozawa et al., 2000, Reymond and Farmer, 1998, Stam et al., 2014, Wu and Baldwin, 2010). Jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) play major roles in the regulatory network underlying plant defence to biotic stresses. The JA and SA pathway tend to be induced by different types of damage. Plants damaged by chewing insect herbivores, such as caterpillars, tend to activate the JA pathway rather than the SA pathway (McCloud and Baldwin, 1997). In contrast, plants damaged by sucking insect herbivores (phloem feeders), such as aphids and whiteflies, tend to induce the SA pathway (Moran and Thompson, 2001). While chewing herbivores bite off and ingest large parts of plant tissue and sap-sucking herbivores hardly make visible mechanical damage, piercing

spider mites, insert their needle-like stylets into the mesophyll cells and feed on the cell contents, resulting in chlorotic spots and eventually loss of photosynthetic capacity (Park and Lee, 2002). JA and its derivatives have been implicated as key components of the defence response to spider mites in e.g. lima bean (Dicke et al., 1999), tomato (Ament et al., 2004), Arabidopsis (Zhurov et al., 2014) and cotton (Miyazaki et al., 2014).

Elevated expression of genes involved in plant defence against herbivores has been reported for several different gene families. For example*, TERPENE SYNTHASES* (*TPSs*) are upregulated by herbivores in various plant species including *AtTPS4* and *AtTPS10* in *Arabidopsis* (Van Poecke et al., 2001, Herde et al., 2008), multiple *TPS* genes in rice (Yuan et al., 2008) and a gene encoding an (*E*)-β-ocimene/(*E,E*)-α-farnesene synthase in cucumber (Mercke et al., 2004). Lipoxygenases (LOXs) are involved in the formation of green leaf volatiles and the precursors of JA and expression of the corresponding genes is induced by herbivore feeding (Gigot et al., 2010). Global profiling of gene expression in spider-mite infested *Arabidopsis* identified 1109 genes that were differentially expressed (differentially expressed gene: DEG) relative to non-infested plants (Zhurov et al., 2014). Using mutants in which JA biosynthesis or JA signalling is disrupted showed that a subset of the induced genes is involved in JA signalling and in the biosynthesis of indole glucosinolates, which were shown to correlate with resistance against spider mites (Zhurov et al., 2014). Recently, spider-mite infested tomato leaves were analysed for transcriptional changes and a comparison between the DEGs in Arabidopsis and tomato revealed a conserved role for genes involved in JA-signalling, and the biosynthesis of phenylpropanoids, flavonoids and terpenoids, in response to this herbivore while specifically in tomato gene sets related to anabolism were suppressed suggesting a possible shift from growth to defence (Martel et al., 2015). Elucidating the expression of genes upon herbivory in plants is a prerequisite to help us gain a better understanding of the processes that play a role in the plant-herbivore interaction.

Cucumber is one of the largest cultivated vegetable crops only preceded by tomato, onion, and cabbage and is thus of major economic and nutritional importance (http://faostat.fao.org). In recent years more genomic resources became available for cucumber such as the *de novo* sequenced genome, a re-sequenced core collection of germplasm, and multiple global expression datasets (Huang et al., 2009, Guo et al., 2010, Qi et al., 2013). In many protected crops, including cucumber, the two-spotted spider mite *Tetranychus urticae* is among the most important pests. Spider mites feed mainly on the abaxial surface of the leaf and penetrate the epidermis to subsequently feed on the content of mesophyll cells. Net photosynthetic rate and total chlorophyll content decrease with progressing spider-mite infestation, up to 95% and 80%, respectively, at high densities (Park and Lee, 2002). Cucurbitacins are bitter triterpenoid compounds that are toxic to many organisms, including spider mites, and occur widely in Cucurbitaceae (Balkema-Boomstra et al., 2003). The only cucurbitacin identified in *Cucumis sativus* is cucurbitacin C and resistance to spider mites could be linked to the constitutive levels of this compound in an F1 generation of a cross between a bitter, spider-mite-resistant cucumber line and a non-bitter,

spider-mite-susceptible line (Balkema-Boomstra et al., 2003). Furthermore, an increase in cucurbitacin C concentration was found when spider mites fed on the cotyledons of a bitter cucumber genotype (Agrawal et al., 1999). Upon infestation with two-spotted spider mites, the volatile blend emitted by cucumber leaves changes and compounds including green leaf volatiles such as (*Z*)-3-hexenyl acetate and various terpenoids such as (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*)-β-ocimene and (*E,E*)-α-farnesene are strongly induced (Takabayashi et al., 1994, Mercke et al., 2004). Moreover, volatile blends induced by spider mites or JA treatment are both attractive to the predator *Phytoseiulus persimilis* (Kappers et al., 2010), implying a role of the JA-mediated signalling pathway in the tritrophic interaction between cucumber, two-spotted spider mites and predatory mites. Analysis of gene expression, using subtractive cDNA libraries enriched in spider-mite up- or down-regulated cDNA fragments, in combination with volatile production data resulted in the identification of two genes encoding a TPS and a LOX, respectively, that are involved in the synthesis of volatiles present in the blend of spider-mite infested cucumber plants (Mercke et al., 2004).

In order to obtain a more detailed understanding of the genes and pathways that are involved in the induced response to two-spotted spider mite feeding in cucumber, we performed a time-course transcriptome profiling of two cucumber varieties Chinese long 9930 (Cl), with bitter foliage due to the presence of cucurbitacin C, and Corona (Co), a non-bitter foliage genotype, upon spider-mite infestation. By analysis of the transcriptional changes we aim to study which signal transduction and biochemical pathways are affected during the first three days of spider-mite infestation.

Results

Visible damage and defence induction in cucumber leaves caused by spider mites

Damage as a result of spider-mite feeding was visible as chlorosis (Fig. 1A) from two days after infestation onwards and reached 5.6 % of the total leaf area in accession Chinese long 9930 (Cl) and 8% in Corona (Co) after three days (Fig. 1B). Mites performed better on accession Co than on accession Cl and more eggs per female were deposited on the former over a time span of 7 days in a leaf disc assay (Fig. 1C).

Spider-mite induced volatile formation on accession Corona has been reported previously (Mercke et al., 2004). To analyse this for accession Chinese long, the volatiles emitted from the infested and non-infested leaves were collected and analysed using GC-MS (Fig. 2A). Green leaf volatiles - including (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate - rapidly increased on the first day post infestation followed by a small decrease in the next two days. The emission of MeSA increased continuously during the three days post infestation (Fig. 2B, inset). Emission of monoterpenoids – including α-pinene, limonene, (*E*)-

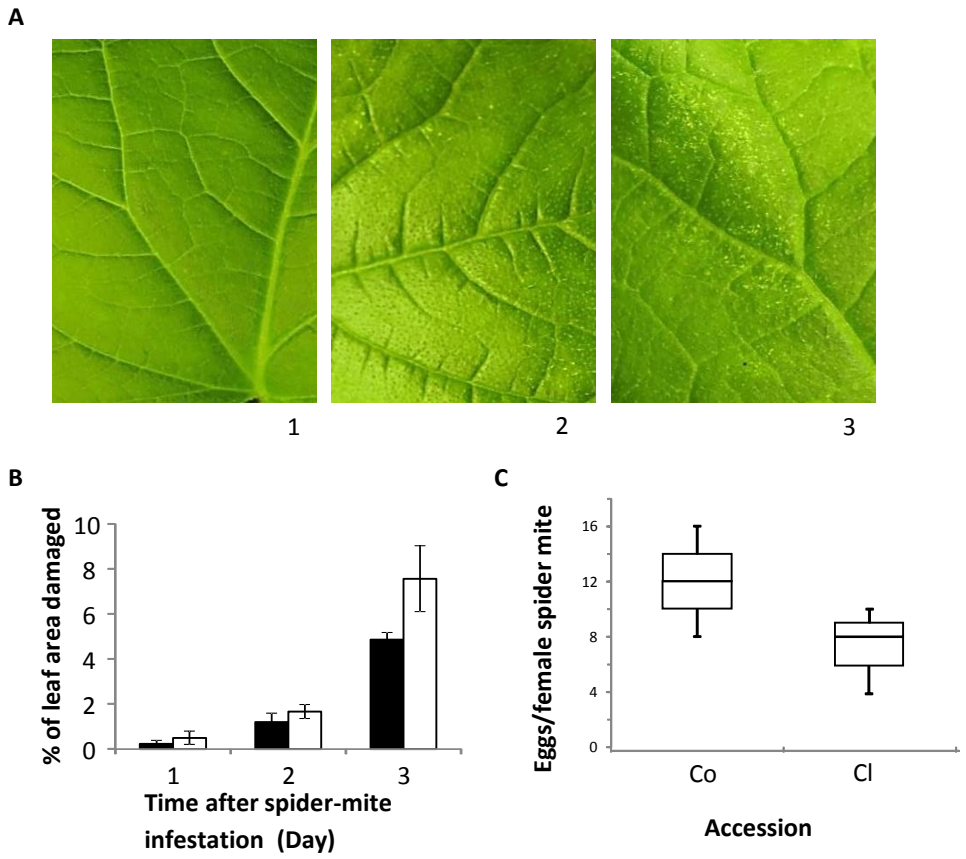


Fig. 1. Spider-mite performance and damage to cucumber leaves. (**A)** Visible damage as chlorotic spots in cucumber accession Chinese long (Cl) after one, two or three days of spider mite infestation. (**B)** Damaged area as percentage of the total leaf area (means ± SD) in bitter accession Chinese long (Cl, ■) and non-bitter accession Corona (Co, □). (**C)** Mean (± SD) number of eggs per female spider mite per day produced in seven days on leaf discs of Chinese long (Cl) or Corona (Co) (*N*=30 leaf discs with single female spider mites).

β-ocimene, an unidentified monoterpene and linalool - gradually increased during the three days post infestation. Sesquiterpene – including (*E*)-β-caryophyllene, α-bergamotene, an unidentified sesquiterpene and (*E,E*)-α-farnesene – emission increased from day 3 onwards. Emission of the homoterpenes DMNT and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) increased strongest, and together they were emitted at even higher levels than the sum of mono- and sesquiterpenoids together (Fig. 2A).

To determine the time frame of early defence responses and hence select time points for RNA-seq analysis, we analysed expression of a gene encoding a LIPOXYGENASE (*Csa2M024440*) and a gene encoding *(E,E)-α-FARNESENE SYNTHASE* (*Csa3M095040*), two genes known to be induced by spider-mite feeding and involved in the biosynthesis of volatile compounds (Mercke et al., 2004). Comparison of non-infested and spider-mite infested Cl leaves showed that transcripts of both genes were not significantly induced within the first 15 hours after the onset of infestation (Fig. 2B). After 24 hours, transcripts of the *LIPOXYGENASE* gene increased, whereas *(E,E)-α-FARNESENE SYNTHASE* expression

showed an increase only at three days after infestation. Because of the minor changes in expression on day 1, we decided to analyse gene expression for both accessions on day 2 and 3 and only for Cl also on day 1.

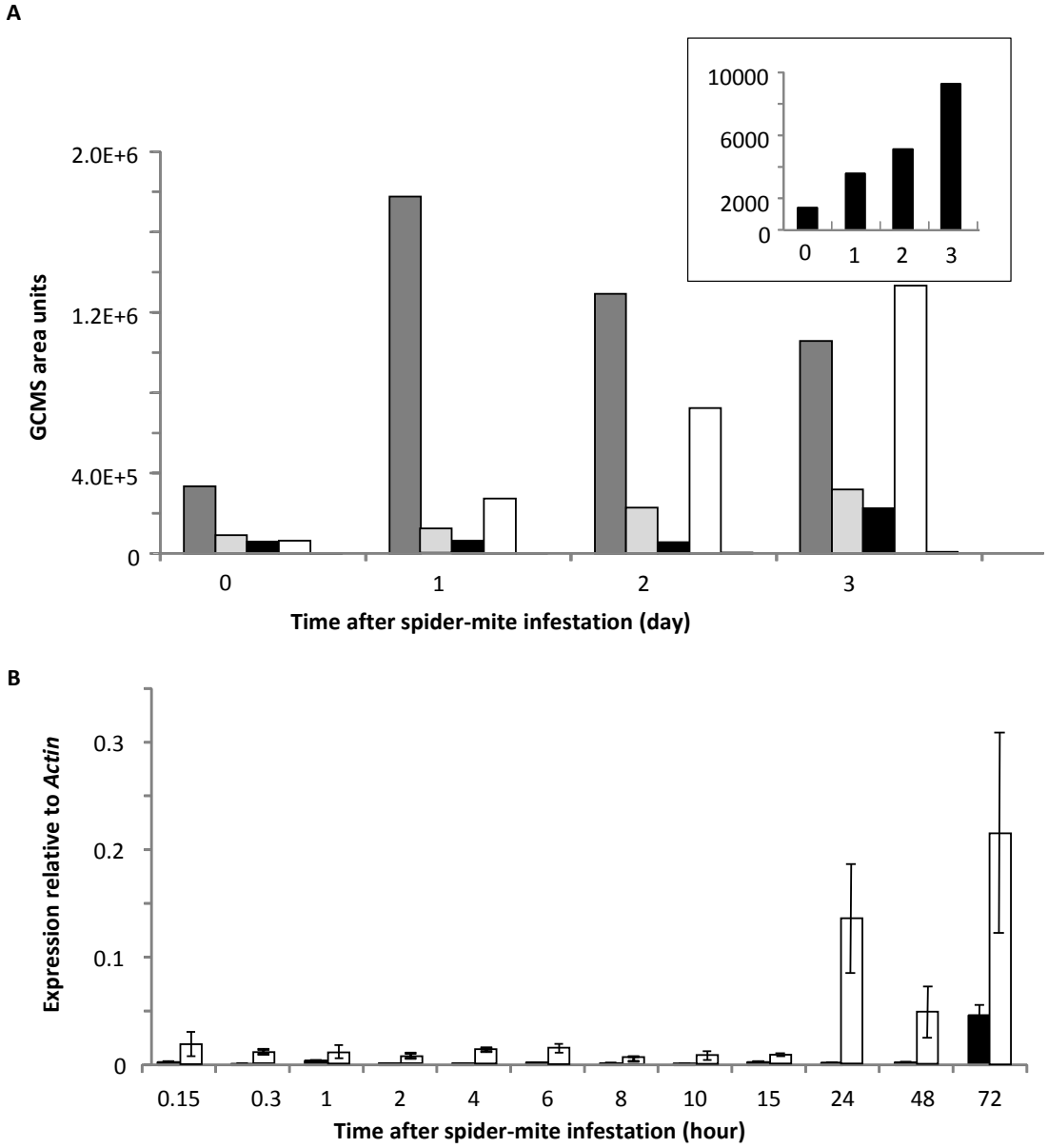


Fig. 2. Defence responses in cucumber plants infested by spider mites. (**A**) Volatiles emitted by cucumber Chinese long leaves that were either non-infested or infested with spider mites for one, two or three days. Dark grey bars (■) represent the sum of green leaf volatiles [(*E*)-2-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate], light gray bars (□) represent the sum of monoterpenes [α-pinene, limonene, (*E*)-β-ocimene, an unidentified monoterpene and linalool], black bars (■) represents the sum of sesquiterpenes [(*E*)-β-caryophyllene, α-bergamotene, an unidentified sesquiterpene and (*E,E*)-α-farnesene] and white bars (□) represent the sum of homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene [DMNT] and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [TMTT]. The inset shows the emission of the benzoate compound methyl salicylate (MeSA). (**B**) Expression of two defence-related genes, *Csa3M095040* encoding a TERPENE SYNTHASE (■) and *Csa2M024440* encoding a LIPOXYGENASE (□) in 12 time points after spider-mite infestation as determined by qRT-PCR. Data represent averages of 3 biological replicates ± SD and were normalized to the transcript level of *Csa6M484600* (*Actin*).

Gene expression profiling in *C. sativus*

Sequencing assessment showed that after removal of adaptor sequences, ambiguous reads and low-quality reads, 12 to13 million high-quality clean reads (99% of the raw data) remained (Fig. S1A). More than 83% of the clean reads could be mapped to the cucumber genome sequence that was obtained for *C. sativus* accession 9930 (Cl, in this study) (Huang et al., 2009). Statistics of the distribution of genes on chromosomes showed that the reads covered most regions of the genome (Fig. S1B). About 68% of the clean reads were mapped to the annotated gene set in the cucumber genome. Around 70% of the annotated genes were covered and saturated by these reads (Fig. S1C). Furthermore, the reads were randomly distributed over the relative positions (5' to 3') within the genes (Fig. S1D).

Since spider mites were not removed from the leaves when these were harvested and frozen, we also aligned the clean reads to the spider-mite genome (Grbic et al., 2011) resulting in max 1% of the reads that could be mapped to the spider-mite genome (data not shown). In summary, the assessment suggests that the obtained sequencing data are representative of the changes in the transcriptome during the time-course experiment. Expression patterns of *Csa1M066550* and *Csa3M095040* (both encoding TERPENE SYNTHASEs) and *Csa2M024440*, encoding a LIPOXYGENASE were analysed by qRT-PCR (Fig. 3). For all three genes both RNA-seq and qRT-PCR analysis indicated a comparable expression pattern during the time span of our experiment and we concluded that the gene expression data we obtained form a good basis for further analysis. We also noticed that the expression pattern of *LIPOXYGENASE*, *Csa2M024440*, differed between different experiments (Fig. 2B, Fig. 3B). However, in both experiments, this *LOX* was strongly induced by spider mites during the first three days of infestation.

Differentially expressed genes upon infestation by spider mites and co-expression patterns

Differentially expressed genes (DEGs) were identified by comparing gene expression at every time point of infestation with that of the non-infested sample for both accessions (Fig. 4A). Two days after the onset of infestation, less than 200 DEGs were identified in accession Cl, whereas in Co more than 400 DEGs were found of which 304 were up-regulated and 108 were down-regulated compared with non-infested leaves (Fig. 4A). For both accessions the number of DEGs strongly increased with progressing infestation to 1006 in accession Cl and 1534 in accession Co after three days. Both accessions shared 505 DEGs three days after infestation, representing half of all DEGs in accession Cl and almost a third in Co (Fig. 4B). Around 60 % of the commonly identified DEGs were found to be up-regulated and 40% were down-regulated in both accessions (Fig. 4B).

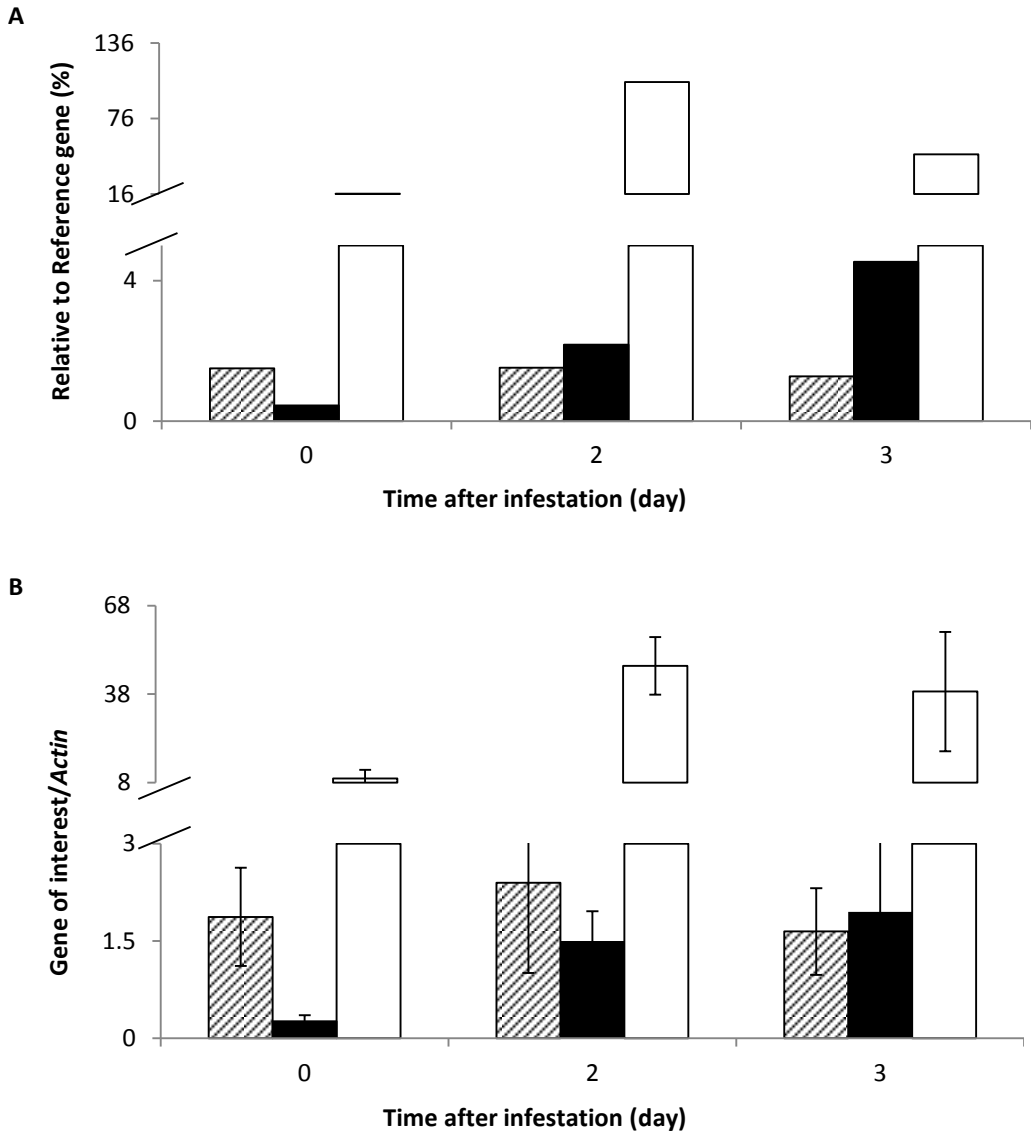


Fig. 3. Validation of RNA-seq transcript data of three selected genes by qRT-PCR. (**A**) Expression of three genes obtained from the RNA-seq data set. (**B**) Expression of the same genes as assessed by qRT-PCR. ▨ *Csa1M066550*; ■ *Csa3M095040*; □ *Csa2M024440*. The cucumber *Actin* gene (*Csa6M484600*) was used as an internal control to normalize expression data using the ΔΔCt method (Bio-Rad, Hercules, CA, USA). Bars represent the average value of three biological replicates ± SD .

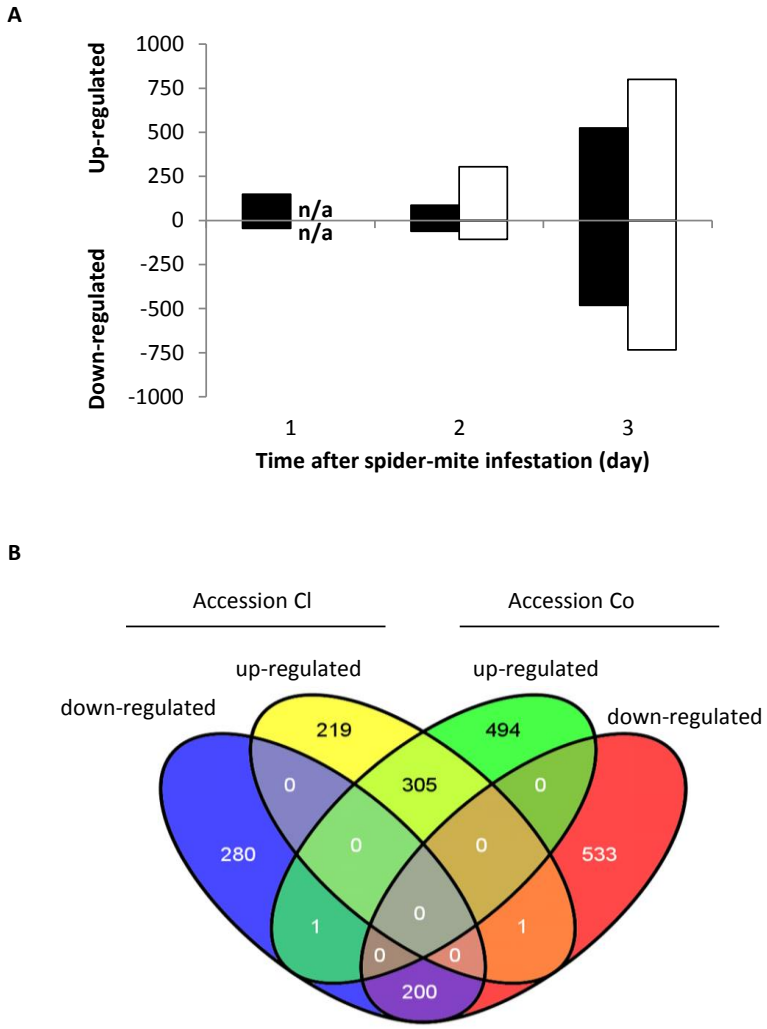


Fig. 4. Transcriptional response to spider-mite feeding in cucumber leaves of accession Chinese long and Corona. (**A)** Differentially expressed genes (log_2 (infested/non-infested) ≥ 1, FDR < 0.01) detected upon spider-mite infestation for one, two or three days in accessions Chinese long (Cl, ■) and two or three days in accessions Corona (Co, □). (**B)** Venn diagram showing overlapping and different DEGs from accessions Chinese long and Corona in leaves that were infested with spider mites for three days.

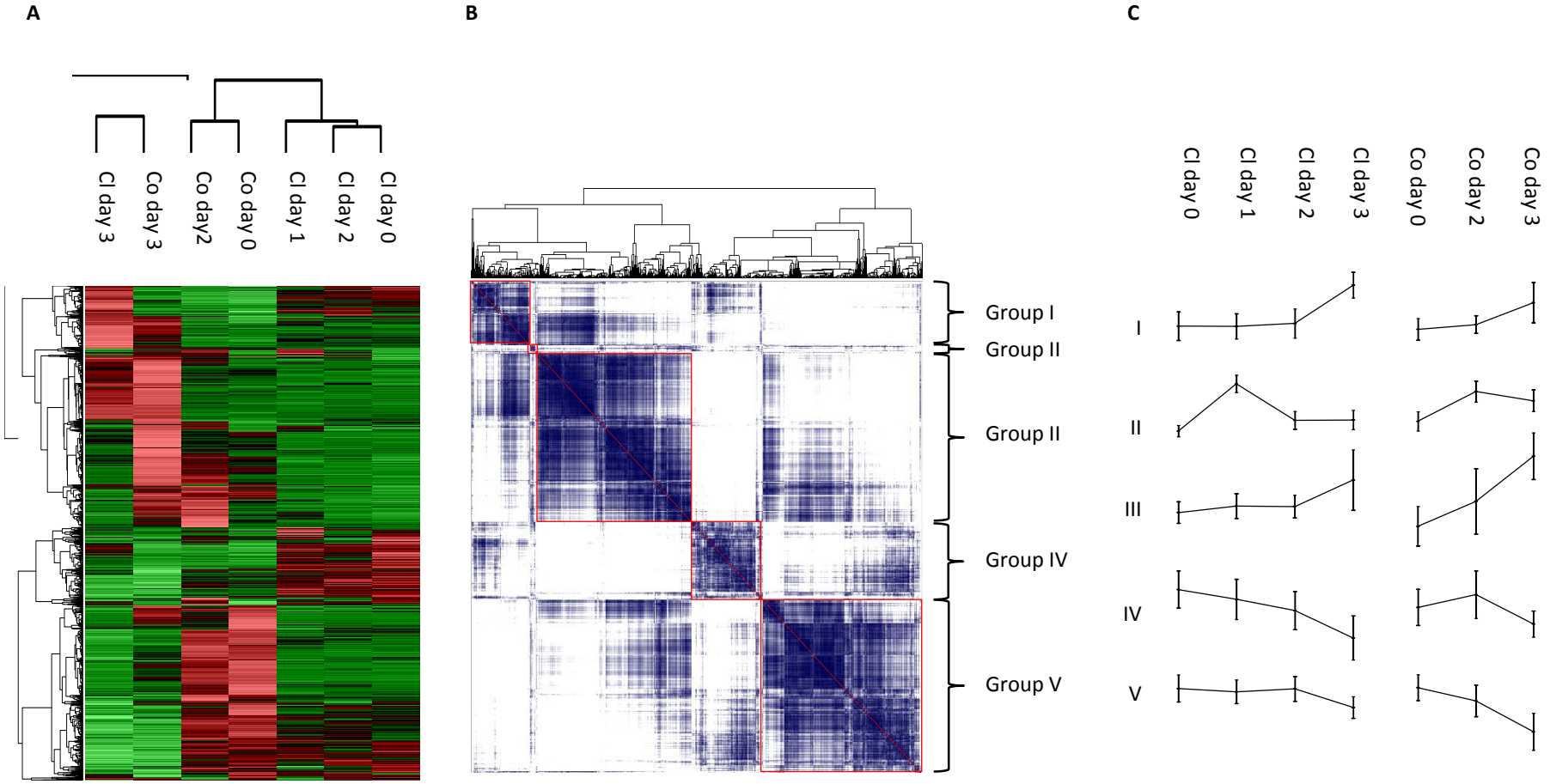


Fig. 5. Expression patterns of DEGs. **(A)** Heat map of 2348 differentially expressed genes in Chinese long (Cl) and Corona (Co) cucumber leaves infested with spider mites for one, two or three days. A colour-coded matrix represents the means of the expression values in RKPM, which have been $\log_2$ transformed and mean-centred. Colour coding: green represents low expression and red represents high expression compared to the average expression of each gene during the infestation. (**B)** Similarity matrix of spider-mite induced DEGs in Chinese long and Corona cucumber leaves. The darker the colour, the higher the similarity of expression patterns. Red rectangles indicate groups of genes that display strongly correlated expression patterns. (**C)** The general expression trend of the genes in each group from **B** for each of the accessions and the different time points

DEGs identified on different days after infestation by spider mites in both accessions Cl and Co together form a collection of 2348 genes. Hierarchical cluster analysis showed that within the first two days of infestation changes in gene expression caused by spider-mite feeding are smaller than the differences in expression between the accessions as on these days the accessions clustered rather than the spider-mite treatment (Fig. 5A). In contrast, three days after the onset of infestation, the herbivores became the major driving force for expression differences and overruled the expression differences between the two accessions. The similarity matrix of gene expression patterns showed that the 2348 DEGs could be divided into five major groups (Fig. 5B). Group I contains 302 genes of which expression increases on day 3 and that are expressed higher in Cl than in Co (Fig. 5C). The second group (II) contains only 29 genes that show quite a different expression pattern compared to all other groups. Expression of these genes was highly up-regulated after one day of infestation in Cl and then decreased again on day 2 and 3 compared to the non-infested control. It is unclear whether Co genes display the same expression pattern, as expression data for Co on day 1 are absent (Fig. 5C). The third group (III) includes 817 genes of which expression continued to increase over three days in both Co and Cl (Fig. 5C). Groups IV and V show that spider-mite infestation does not only result in upregulation of gene expression. These groups contain 371 and 829 genes, respectively, of which expression is suppressed compared with the non-infested control (Fig. 5C). In group IV this suppression is strongest in accession Cl, while in group V it is strongest in Co.

Enrichment in function of differentially expressed genes

In order to understand which biological processes and functions were activated or repressed in cucumber after spider-mite infestation, we screened for enriched Gene Ontology (GO) terms in the DEGs within each group compared to the whole set of genes annotated with GO terms (Table 1, Table S2). As discussed above, groups I, II and III (Fig. 5) represent genes that are upregulated by spider-mite infestation. GO-term enrichment of DEGs in group I showed that ‘photosynthesis’, ‘translation’, ‘response to red light’, ‘response to far red light’, ‘generation of precursor metabolites and energy’ and ‘photosynthesis and light reaction’ were the six enriched biological processes up-regulated early during spider mite infestation. Molecular functions of these genes are classified as ‘electron carrier activity’ and ‘structural constituent of ribosome’ whereas cellular component analysis showed that the GO-terms related to ‘photosynthetic membrane’, ‘photosystem’, ‘thylakoid’, ‘plastid’, ‘chloroplast part’, ‘nucleolus’ and ‘ribosome’ were significantly enriched (Table 1). In group II only molecular function ‘oxidoreductase activity’ was enriched probably due to the small size of this group (only 29 genes). Classifying group III genes to biological processes resulted in enrichment of GO-terms including ‘response to stress’, ‘aromatic amino acid family catabolic process’, ‘L-phenylalanine catabolic process’, ‘response to other organisms’ and 19 other terms (Table 1). In this category, 203 genes were classified as ‘response to stimulus’, including eight genes

Table 1. Gene Ontology terms enriched in the different groups of genes that were differentially expressed (DEGs) between spider mite infested and control plants of cucumber accessions Chinese long (Cl) and Corona (Co). Enrichment analysis was performed using a hypergeometric test (Rivals et al., 2007). The *P* value was calculated using the Hochberg [FDR, Reiner et al., (2003)] multi-test adjustment, taking FDR ≤ 0.05 as a threshold.

Group	Characteristic gene expression profile		GO-term enrichment analysis		
	Cl	Co	Molecular function	Biological process	Cellular localization
Group I	drastic increase on day3	increase on Day 3	structural constituent of ribosome	photosynthesis	photosynthetic membrane
			structural molecule activity	translation	thylakoid part
			electron carrier activity	response to red light	plastid thylakoid membrane
				response to far red light	chloroplast thylakoid membrane
				generation of precursor metabolites and energy	thylakoid membrane
				photosynthesis, light reaction	thylakoid
					cytosolic ribosome
					plastid thylakoid
					chloroplast thylakoid
					organelle subcompartment
					*34 terms in total
Group II	drastic increase on day 1	drastic increase on day 2	oxidoreductase activity		
Group III	increase on Day 3	drastic increase on day 3	ammonia-lyase activity	response to stress	
			phenylalanine ammonia-lyase activity	aromatic amino acid family catabolic process	
			carbon-nitrogen lyase activity	L-phenylalanine catabolic process	
				response to other organism	
				response to biotic stimulus	
				response to stimulus	
				response to chemical stimulus	
				response to chitin	
				defense response	
				response to wounding	
				* 24 terms in total	
Group IV	drastic decrease on day 3	decrease on day 3			apoplast
					external encapsulating structure
					cell wall
					extracellular region
					cytosolic ribosome
Group V	decrease on day 3	drastic dcrease on day 3		phenylpropanoid metabolic process	
				flavonoid metabolic process	

involved in the 'jasmonic acid mediated signalling pathway', 17 genes in 'response to JA stimulus', 16 genes involved in 'response to chitin' and 22 genes involved in 'response to wounding' (Table 1, Table.S2). GO-term analysis did not yield any enrichment of cellular components and for molecular functions only 'phenylalanine ammonia-lyase activity' was found enriched in group III (Table 1). Groups IV and V contain DEGs suppressed by spider mites. In group IV cellular components of 'cell wall', 'cytosolic ribosome' and 'apoplast' were enriched but no GO-terms for biological processes nor for molecular functions were significantly enriched. Finally, DEGs in group V were enriched for 'phenylpropanoid- and flavonoid metabolic processes'.

Influenced KEGG pathways in cucumber in response to spider-mite feeding

To obtain more insight in how different metabolic pathways that were found enriched in the GO-term analysis interact with each other, spider-mite induced DEGs were mapped to the KEGG pathway database (Kanehisa et al., 2014) (Fig. S2). Three days after the onset of mite infestation several pathways were significantly influenced, including 'ribosome' (*P*=9.9E-07, 18 up and 15 down regulated genes), 'protein export' (*P*=2.0E-04, 7 up, 2 down), photosynthesis (*P*=4.0E-04, 6 up, 3 down), the phagosome pathway (*P*=0.01, 7 up, 3 down) and sesquiterpenoid and triterpenoid biosynthesis (*P* =0.03, 2 up, 2 down). Only just not significant were diterpenoid biosynthesis (*P*=0.07, 4 up, 3 down) and terpenoid backbone biosynthesis (*P*=0.07, 4 up, 1 down). Pathways that were significantly activated already on day one after infestation included phenylpropanoid biosynthesis (*P*=9.8E-17, 20 up, 6 down), phenylalanine metabolism (*P*=1.6E-13 6 up, 2 down), linoleic acid metabolism (*P*=1.1E-10, 10 up, 0 down) and α-linolenic acid metabolism (*P*=1.5E-06, 14 up, 3 down).

Spider-mite feeding affects JA and SA signalling in cucumber

As the 'response to jasmonic acid stimulus' was enriched in the GO-term analysis (Table S2), we had a closer look at the expression of the genes in this category in both accessions at different time points (Fig. 6A). This set consists of 17 genes including five transcription factors, three *LIPOXYGENASES*, *ALLENE OXIDE SYNTHASE* and the gene encoding the JASMONATE ZIM DOMAIN PROTEIN. Generally, all genes were up-regulated by infestation with spider mites, but their expression patterns were not identical. Most of these genes were highly induced on day 3, but more transcripts of the three *LIPOXYGENASE* genes were found on day 1. In addition to 'response to jasmonic acid stimulus', the GO-term 'salicylic acid metabolic process' was also enriched on day 3 and therefore we had a detailed look at the expression patterns of five DEGs in this category (Fig. 6A). All five genes are annotated as *PHENYLALANINE AMMONIA-LYASE* (*PAL*) and their expression patterns are similar. In

accession Cl these genes were rapidly induced one day post infestation but suppressed again in the next two days. In accession Co, they were up-regulated on day 2 and 3. We also analysed the expression of the cucumber homologs of *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) and *PHYTOALEXIN DEFICIENT 4* (*PAD4*), two genes that interact and function up- and downstream of SA (Rietz et al., 2011). No significant expression changes were detected for both genes in Cl during the first three days of infestation. In Co, expression levels of both genes decreased on day 3 to approximately half of the initial expression level. In contrast, the cucumber homolog of *PATHOGENESIS RELATED 1* (*PR1*), which is known to be up-regulated by SA-signalling (Cameron et al., 1999), was upregulated 2.6- and 2.2-fold on day 3 in accessions Cl and Co, respectively. Except for JA and SA, no other phytohormone-related GO-terms were enriched in the spider-mite induced DEGs.

To further evaluate the involvement of JA and SA in the response of cucumber to spider mites we analysed JA and SA dynamics in spider-mite infested cucumber leaves (Fig. 6B). JA concentrations increased transiently after the introduction of spider mites, reaching a 7-fold increase compared to non-infested leaves one hour after the onset of infestation (Fig. 6B). Two hours after the start of infestation, JA concentrations were back at base-line level and then again slowly increased to 2.5-fold compared to non-infested leaves after five days of infestation. SA concentrations showed an, although not significant, opposite trend as a transient 2-fold decrease was detected one hour after the onset of infestation.

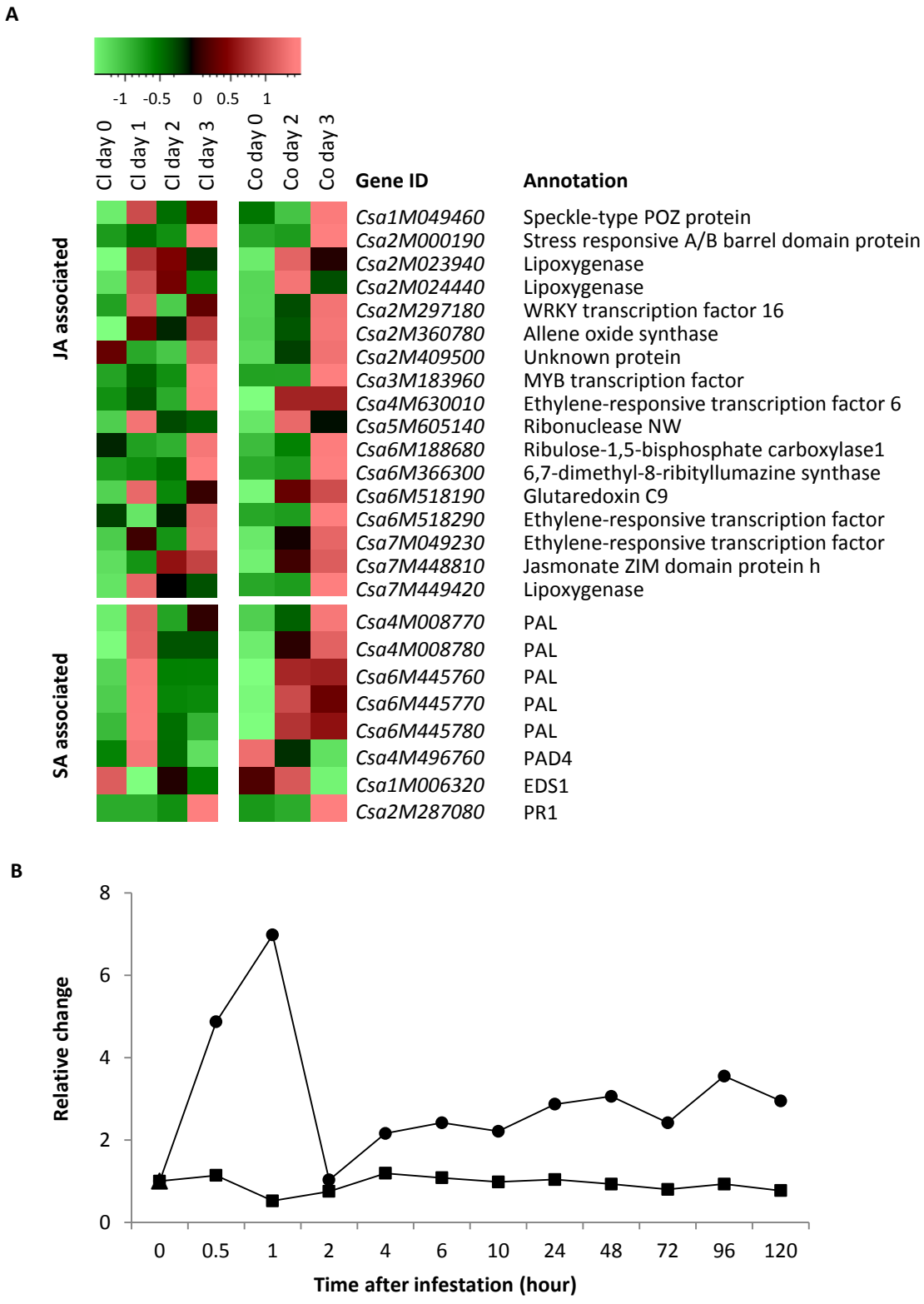


Fig. 6. Changes in expression of genes associated with jasmonic acid and salicylic acid signalling, and in the content of these two phytohormones in cucumber leaves during spider-mite infestation. (**A)** Relative expression of JA- and SA-associated genes in accessions Chinese long (Cl) and Corona (Co) infested by spider mites for one (only for Cl), two and three days. Expression levels were normalized to the standard deviation of each gene during the infestation. Colour coding: green represents low expression level and red represents high expression level. (**B)** Relative changes in JA (solid circles)and SA (solid squares) compared to the level in non-infested leaves.

Spider mites upregulate the expression of genes associated with photosynthesis during the first days of infestation

GO term analysis revealed that genes related to photosynthesis were upregulated by spider mites early during infestation. We checked the putative functions and expression patterns of the genes present in this category (Fig. 7A). At least 25 DEGs were involved in photosynthesis, including eight genes encoding CHLOROPHYLL A-B BINDING PROTEIN (CAB), the subunit for the light-harvesting chlorophyll protein complex (LHC). Furthermore, genes encoding subunits of photosystem II (PsbA, PsbQ, PsbY and Psb28) and photosystem I (PsaD, PsaE and PsaK), PLASTOCYANIN, FERREDOXIN and F-TYPE ATPASE were upregulated early during spider-mite infestation in both accessions. Transcripts of most of these genes gradually increased during the first three days of infestation in leaves of accession Cl. For example, transcripts of *Csa2M350230* (encoding photosystem II PsbY) were 1067, 1050, 1070 and 1599 RPKM in Cl on day 0, 1, 2 and 3, resulting in a 1.5-fold increase after three days of infestation compared to non-infested plants. A gene encoding photosystem II Psb28 (*Csa6M087980*) was upregulated 2.3-fold on day 3 of infestation compared to day 0. Similarly, almost all genes in this pathway were 2-fold or more up-regulated in spider-mite infested leaves of accession Co on day 3 of infestation compared to non-infested plants. In the published data on spider-mite induced transcriptional changes in tomato (Martel et al., 2015), we found a similar pattern, albeit with different temporal dynamics. Eleven genes encoding CAB were induced by spider mites at three and six hours after the onset of infestation compared with one hour after infestation (Fig. 7B). Upon prolonged infestation for 12 and 24 h, however, expression decreased again. In our experiment this decrease was not visible yet even after three days of infestation. In tomato, the gene encoding Ferredoxin is also upregulated during infestation, showing dynamics that are very similar to the changes in cucumber.

We also had a more detailed look at differentially regulated genes encoding proteins involved in sucrose biosynthesis and transport such as *HEXOKINASE*, *SUSY (SUCROSE SYNTHASE)* and *SUT (SUCROSE TRANSPORTER)* in cucumber during the infestation of spider mites (Fig. S3). Interestingly, unlike the upregulated photosynthesis-associated genes, sucrose-associated genes were generally downregulated during the first three days of infestation, especially in accession Cl.

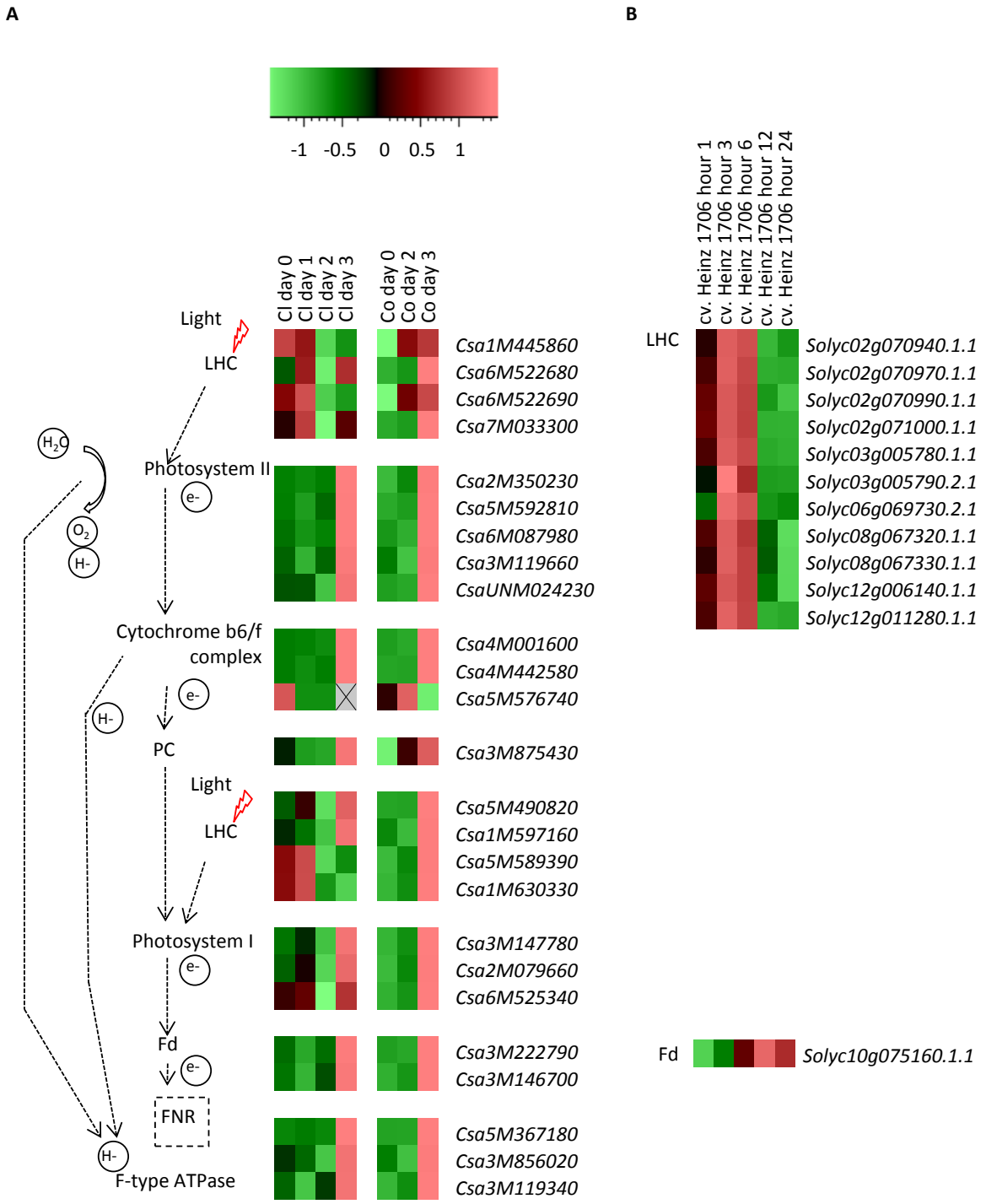
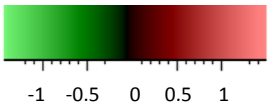
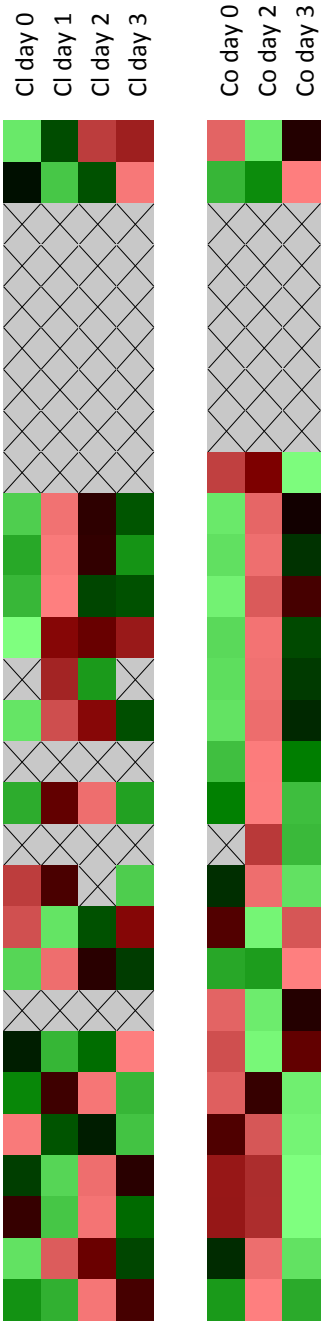


Fig. 7. Photosynthesis-associated genes respond to spider-mite herbivory. (**A**) The pathway of photosynthesis (left) and the expression pattern of the associated genes in cucumber accessions Chinese long (Cl) and Corona (Co) infested by spider mites. LHC: LIGHT-HARVESTING CHLOROPHYLL PROTEIN COMPLEX; Fd: FERREDOXIN; PC: PLASTOCYANIN; FNR: FERREDOXIN-NADP^{+} REDUCTASE. (**B**) The expression pattern of photosynthesis-associated genes in tomato cv. Heinz 1706 infested by spider mites (Martel et al., 2015). Colour coding: green represents low expression and red represents high expression compared with the average expression of each gene during the infestation.





Gene ID	DEGs	Type	Symbol
Csa6M424000		I	*CsLOX9*
Csa6M500530			
Csa4M288080		II	*CsLOX21*
Csa4M287020		II	
Csa4M286990		II	*CsLOX13*
Csa4M286380			*CsLOX7 **
Csa2M027460		I	*CsLOX3*
Csa2M023870		I	*CsLOX6*
Csa4M288100		II	*CsLOX18,20*
Csa4M286970	X	II	
Csa4M286940	X	II	*CsLOX17*
Csa4M286960	X	II	*CsLOX16*
Csa2M026940	X	I	
Csa4M287530	X	II	*CsLOX14*
Csa2M024440	X	I	
Csa5M141160			
Csa2M023880	X	I	*CsLOX1*
Csa4M287550		II	*CsLOX15*
Csa2M023900		I	*CsLOX2*
Csa7M374630		I	*CsLOX11*
Csa7M449420	X	II	*CsLOX22*
Csa4M288070		II	*CsLOX12*
Csa6M500520			
Csa2M023850		I	*CsLOX8*
Csa7M374620		I	
Csa2M023340		I	*CsLOX10*
Csa5M590080		II	*CsLOX23*
Csa2M028480	X	I	*CsLOX4*
Csa4M288610		II	*CsLOX19*

Fig. 8. Expression pattern of genes encoding lipoxygenases (*CsLOX*s) in spider-mite infested leaves of cucumber accessions Chinese long (Cl) and Corona (Co). Expression levels were normalized to the standard deviation of each gene during infestation. Colour coding: green represents low expression and red represents high expression compared with the average expression of each gene during the infestation. Genes were arranged by hierarchical clustering of the expression (Pearson correlation was used to calculate the pairwise distance and UPGMA was used to summarize the distance between clusters). Grey areas indicate that no transcript was detected for that time point. Classification of LOXs based on the presence of a putative targeting signal peptide (Type I or type II LOX) as determined by phylogenetic relationship (Fig. S5). The DEGs identified during infestation of spider mites are indicated by "X". **CsLOX5* was not detected and *CsLOX7* is not annotated as a *LOX* gene in the annotation of cucumber genome version 2 [Li et al. (2011), http://www.icugi.org/, version 2].

LOX genes upregulated by spider mites

Multiple *LOX* genes were rapidly and strongly induced by spider mites in our study. For example, among the top 15 strongest induced genes in accession Cl after one day of infestation, five genes encode lipoxygenases. We therefore studied the expression pattern of the whole gene family. According to Huang et al. (2009), the *CsLOX* gene family has 23 members. However, using the updated annotation information from the cucumber genome project (http://www.icugi.org/, version 2), we found 28 putative gene members (Fig. 8). According to the sequence similarity, 11 of them encode putative Type I LOXs (in the same subclade as AtLOX1 and AtLOX5, Fig. S4), lipoxygenases without a targeting signal peptide (Feussner and Wasternack, 2002). Thirteen genes putatively encode Type II LOXs (in subclade with AtLOX 2-4 and AtLOX6, Fig. S4) with putative chloroplast targeting signal peptide sequence (Feussner and Wasternack, 2002). The proteins encoded by the remaining three genes could not be classified as either Type I or Type II but form a separate subclade.

In total, nine *CsLOXs* were identified as DEGs during the infestation by spider mites either in Cl or Co (Fig. 8). The expression of these genes was generally strongest up-regulated on day 1 or day 2 in accession Cl or on day 2 in Co, followed by a decrease in expression on day 3. Among them five are type I and the other four are type II. All nine *CsLOXs* were induced at least 2-fold in Cl one day after infestation and in Co two days after infestation. Some *CsLOXs* were quite strongly up-regulated. For example, expression of *Csa2M024440* (Type I) increased 79-fold on day 1 compared with non-infested plants in Cl and 62- and 35–fold on day 2 and 3, respectively, in Co. Of six *CsLOX*s no expression was detected at any time point in both accessions.

Genes involved in biosynthesis of terpenoids regulated by spider mites

GO-term analysis for DEGs from different days after infestation revealed terpenoid related terms including “terpene metabolic process” and “sesquiterpenoid metabolic process” enriched three days post infestation (data not shown). Furthermore, pathways of sesquiterpenoid, triterpenoid biosynthesis, terpenoid backbone biosynthesis, limonene and pinene degradation were found enriched among DEGs in leaves that were infested with mites for three days (Fig. S2). Therefore we analysed the expression patterns of these DEGs in the processes of infestation in more detail (Fig. 9). Five DEGs were assigned the GO term of terpenoid backbone biosynthesis. Despite the enrichment of ‘sesquiterpene metabolic process’, the gene *Csa7M029390* encoding HMG-CoA reductase (3-hydroxy-methylglutaryl coenzyme A reductase) which catalyses the rate limiting step in the cytosolic mevalonate pathway that is supposed to produce the substrate for sesquiterpene and triterpene biosynthesis was down-regulated in both accessions during the infestation. In contrast, *Csa1M420340* encoding DXP-synthase (1-deoxy-D-xylulose-5-phosphate synthase) which

catalyses the rate limiting enzymatic stepin the plastidic MEP/DOXP pathway (Estevez et al., 2001) that in principle supplies the substrate for mono- and diterpene biosynthesis was upregulated. *Csa6M487640* and *Csa7M211090* both encode GPP synthase and were upregulated in Cl during infestation. In Co this was true only for *Csa7M211090*. Expression of *Csa2M29988* - encoding a putative monoterpene synthase - and *Csa3M09504* - encoding a putative sesquiterpene synthase - was not or hardly detected in non-infested plants and was up-regulated during the three days of infestation in both Cl and Co. A gene (*Csa6M185830*) encoding isoprenyl transferase which may play a role to form dehydrodolichol-PP was also upregulated in both accessions. Three of the seven *P450* genes, which were mapped into the pathway of limonene and pinene degradation but may be involved in the conversion of the terpene backbone into oxidised terpenoids, were up-regulated in both accessions after infestation. However, three of the remaining *P450*s are suppressed by the infestation, while the last one is up-regulated in accession Cl but down-regulated in accession Co.

Spider mites suppress genes associated with biosynthesis of cucurbitacin C

Because cucurbitacin C has been implicated in the resistance of cucumber to herbivores, the expression of genes involved in the biosynthesis of cucurbitacin C was analysed in more detail. Nine genes functional in this pathway were recently identified (Shang et al., 2014) and transcripts of all except one were detected in our RNA-seq data. The expression of these eight genes was down regulated after spider mite infestation in both the bitter accession Cl and the non-bitter accession Co (Fig. 10A). Expression of the *Bi* gene (*Csa6M088690*), known to confer bitterness and recently shown to encode OXIDOSQUALENE CYCLASE, was not detected in our RNA-seq experiment. However, qRT-PCR analysis for this gene revealed that transcripts are indeed present in Cl and decrease after infestation (Fig. 10B). Furthermore, in an independent experiment the amount of cucurbitacin C in leaves of accession Cl that were infested with spider mites showed a negative (though not significant) trend with progressing infestation during three days (Fig. 10C, D), while no cucurbitacin C was detected in Co.

The genes encoding transcription factors that display co-expression with cucurbitacin C associated genes were analysed. Based on the expression patterns in cucumber during infestation of spider mite, infestation of downy mildew (Adhikari et al., 2012) and in different organs in unchallenged condition (Li et al., 2011), seven of the nine cucurbitacin associated (Fig. S5) genes were found co-expressed with a gene encoding a bHLH type transcription factor (*Csa1M051740*). This transcription factor was assigned GO term response to gibberellin stimulus (GO:0009739).

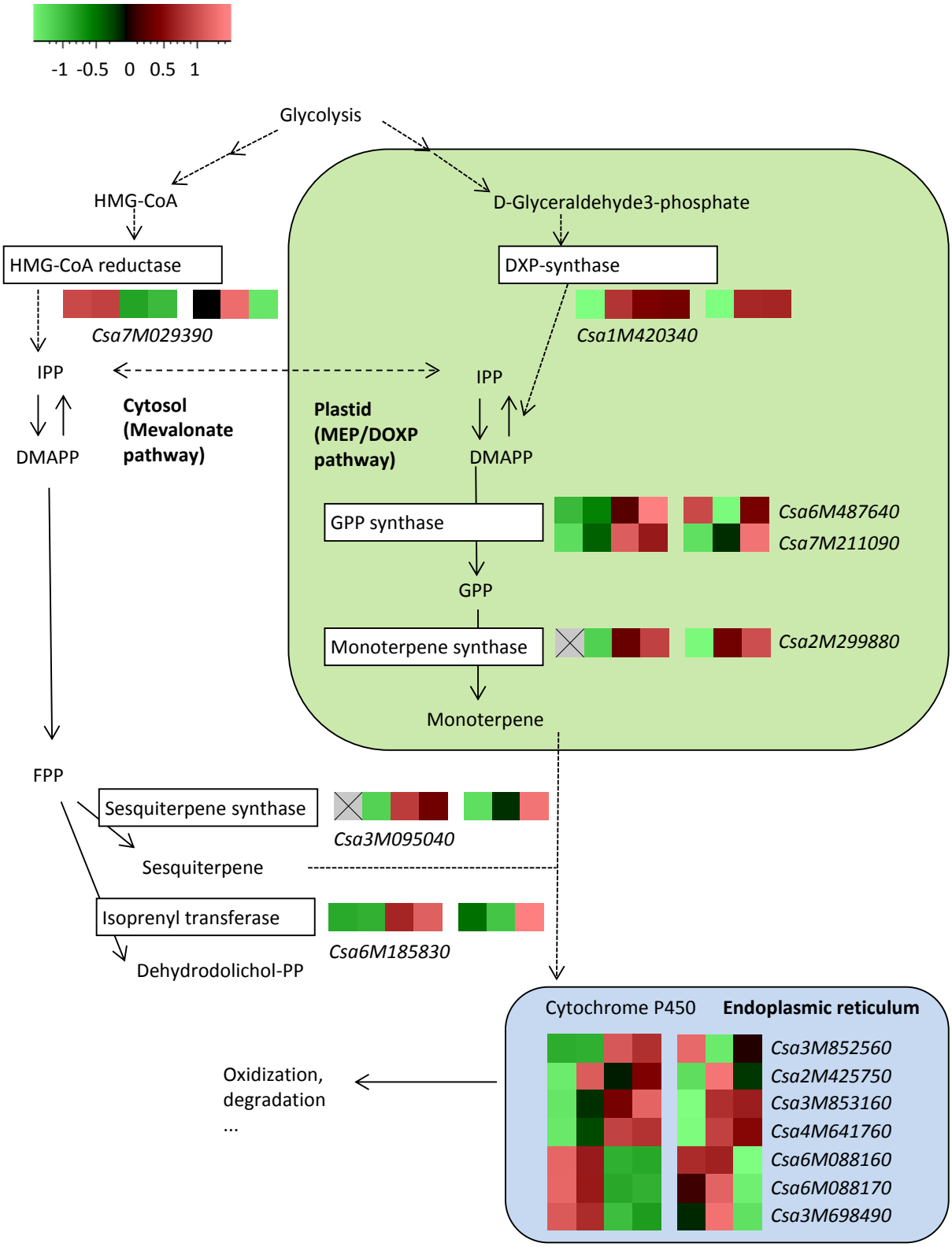


Fig. 9. Terpenoid biosynthesis and relative expression of associated genes in accessions Chinese long (Cl) and Corona (Co) infested by spider mites for different time periods. Expression levels were normalized to the standard deviation of each gene during the infestation. Colour coding: green represents low expression and red represents high expression compared with the average expression of that gene during the infestation. For each gene, the seven blocks represent expression levels in accession Chinese long on day 0, 1, 2, 3 and accession Corona on day 0, 1, 3 from left to right.

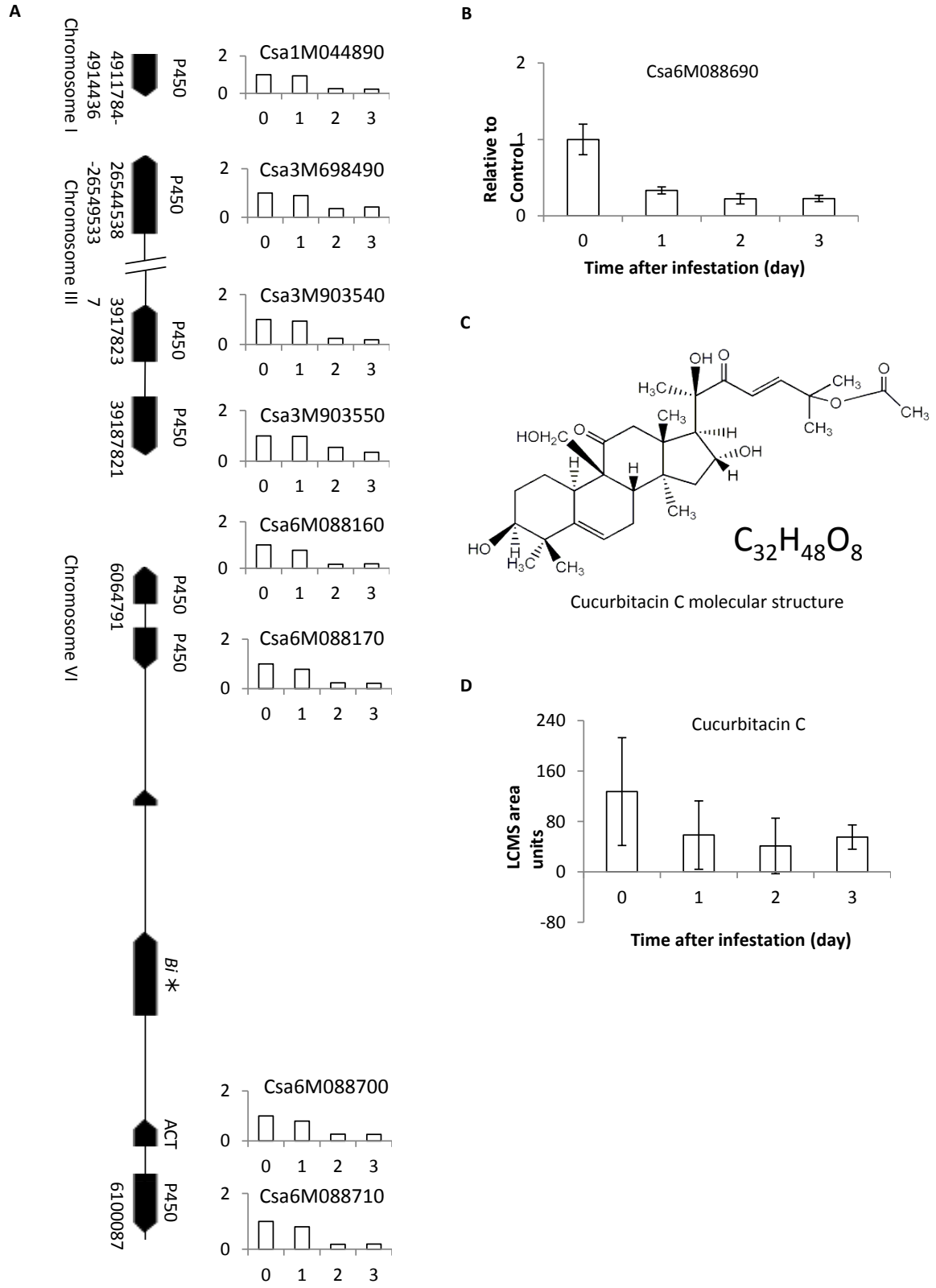


Fig. 10. Relative expression of genes associated with biosynthesis of cucurbitacin C in spider-mite infested cucumber leaves. **(A**) Transcripts of genes involved in cucurbitacin C biosynthesis (Shang et al., 2014) in leaves of accession Chinese long after infestation with spider mites for one, two or three days.(Shang et al., 2014) (**B)** qRT-PCR analysis of *Bi* (*Bitter*) gene (*Csa6M088690*) transcripts in spider-mite infested leaves of accession Chinese long. (**C)** Cucurbitacin C. (**D**) Concentration of cucurbitacin C in cucumber Chinese long leaves that were infested by spider mites for one, two or three days or were left untreated.

Discussion

Plants are constantly challenged by pathogens and herbivores and have developed an arsenal of defence responses to try to protect themselves. The defence of plants consists of multiple layers that interplay which each other. Next to basal physical and chemical barriers that hinder attackers to invade, plants have evolved a wide range of inducible defence mechanisms that are triggered upon attack (Bari and Jones, 2009). Recognition of the type of attacker leads to a transient reconfiguration of the transcriptome of the plant in such a way that, amongst others, the metabolome is altered to try to cope with the attacker. In turn, however, herbivores try to suppress the defence of the plant. In this study, we provide an analysis of genes affected by early spider-mite herbivory on cucumber foliage. In the first three days of infestation by spider mites, we identified 2348 DEGs that were either positively or negatively responsive to mite feeding. Upon annotation of the functions of these DEGs according to gene ontology (GO) and KEGG pathway databases, we found that genes involved in JA signalling, photosynthesis, biosynthesis of terpenoids and encoding LIPOXYGENASEs were upregulated. Genes associated with flavonoids and, surprisingly, genes associated with the biosynthesis of cucurbitacin C, which is an important anti-herbivore secondary metabolite in cucumber (Balkema-Boomstra et al., 2003), were suppressed during the first three days of spider mite infestation.

JA is involved in cucumber response to spider mites

From our RNA-seq data, genes involved in the JA-mediated signalling pathway and the response to JA stimulus were found to be induced upon spider-mite infestation. These genes encode proteins with various functions including JAZ2, LIPOXYGENASE, WRKY and MYB transcription factors, allene oxide synthase and riboflavin synthase. Furthermore, a transient 7-fold increase in JA was detected after one hour of spider-mite infestation followed by a gradual increase with progressing infestation. The activation of JA signalling by spider mites has been widely observed in plants species such as *Arabidopsis* (Zhurov et al., 2014), lima bean (Hopke et al., 1994, Ozawa et al., 2000, Dicke et al., 1999) and tomato (Ament et al., 2004, Agrawal et al., 2002). Transcriptional changes in spider-mite infested *Arabidopsis* leaves suggest JA as the major hormone mediating inducible defence responses (Zhurov et al., 2014). In tomato, genes involved in the jasmonate pathway responded to spider-mite infestation within one day and emission of volatile terpenoids and attractiveness to predators significantly increased after 4 days (Kant et al., 2004). Comparison of DEGs in the tomato *def-1* mutant, which is mutated in the ability to accumulate JA in response to wounding and herbivory (Howe et al., 1996), showed that approximately 95% of DEGs 24 hours after infestation by spider mites are dependent on JA (Martel et al., 2015). Upon spider-mite infestation for seven days, cucumber leaves emitted various volatiles not emitted by non-infested plants and JA treatment resulted in a virtually similar volatile blend

(Kappers et al., 2010). These volatiles include green leaf volatiles and terpenoids that are produced in different proportions by different accessions, which results in differences in attractiveness towards predatory mites (Kappers et al., 2011).

The emission of methyl salicylate (MeSA) is the result of methylation of SA (Wildermuth, 2006) and is produced from accumulating SA. MeSA is a significant component of floral scents of many species, and of the volatile blend of some herbivore-attacked vegetative tissues (Van Den Boom et al., 2004). Interestingly, MeSA is not induced in lima bean by JA treatment (Dicke et al., 1999), but it is induced by JA in tomato (Ament et al., 2004). It was shown to be involved in the attraction of pollinators as well as natural enemies (Van Poecke et al., 2001, Zhu and Park, 2005) and in cucumber MeSA is emitted in small amounts in some genotypes after spider mite infestation while it is absent in the blend of others (Kappers et al., 2010). In Arabidopsis, *BSMT1* encodes an SA carboxy-methyltransferase and expression in leaves is upregulated by JA and herbivory (Chen et al., 2003). In cucumber three *BSMT*1 homologs were found but none of them was induced in either Cl or Co, although MeSA was detected among the volatiles emitted from cucumber leaves infested by spider mites. Only one SA related GO-term, 'salicylic acid metabolic process', was found enriched one day after the onset of spider-mite infestation. The DEGs responsible for this enrichment comprise five putatively annotated genes encoding phenylalanine ammonia-lyase (PAL) which catalyses the first step of the formation of a wide variety of natural products with a phenylpropanoid skeleton from L-phenylalanine, including salicylic acid (Chen et al., 2009). Accumulation of *PAL* transcripts was found to be increased after pathogen infestation, wounding and UV light (Dixon and Paiva, 1995). However, PAL is also involved in many other pathways and does not specifically indicate activity of the SA pathway. Furthermore, expression of SA marker genes *EDS1* and *PAD4* did not increase in Cl during the infestation; moreover, they were even downregulated in Co. In contrast, *PR1* , a well-known SA-responsive marker gene (Cameron et al., 1999) was up-regulated in both accessions by spider mites. Changes in concentration of SA in cucumber during the infestation of spider mite were also minor (0.5- to 1.2-fold relative to non-infested plants). Endogenous SA increased and the expression of downstream pathway genes was induced in lima bean upon infestation by spider mites (Ozawa et al., 2000). SA also accumulated in Arabidopsis leaves that were infested with a very high density of spider mites (Zhurov et al., 2014). Taken together, we only found weak indications that SA signalling is induced by spider mites during the first days of infestation.

Photosynthesis related genes are upregulated by spider mites

We observed clear chlorosis in spider-mite damaged leaves but interestingly, at least 14 genes involved in photosynthesis were up-regulated during the first three days of infestation in both accessions (Fig. 7, Table S2). Most of these genes encode different subunits of photosystem I and II, or units for the light-harvesting complex such as chlorophyll a/b

binding proteins. Spider mites ingest the full cell content, including chloroplasts (Park and Lee, 2002). Damage caused by this herbivore eventually results in reduced chlorophyll levels and hence impairs the ability of the plant to photosynthesize (Iatrou et al., 1995). Moreover, the damage causes dehydration of epidermal cells and closure of stomata leading to decreased gas exchange and hence also indirectly negatively affects photosynthesis (Bueno et al., 2009, Bilgin et al., 2010). Downregulation of photosynthesis is a common response to biotic stresses including herbivory and photosynthetic rate reduction caused by spider mites has been reported in multiple plant species such as soybean (Bueno et al., 2009), apple (Ferree and Hall, 1980) , and strawberry (Sances et al., 1981). In contrast, the salivary secretions of a herbivore mirid bug enhanced photosynthesis in *Nicotiana attenuata* (Halitschke et al., 2011). Photosynthesis related GO-terms were also enriched in the transcriptome profiling of spider-mite infested tomato within 24 hours (Martel et al., 2015), including 11 genes that encode chlorophyll a/b binding proteins of which expression was upregulated at three and six hours after the onset of infestation followed by downregulation at later time points (Martel et al., 2015).Temporal upregulation of genes associated with photosynthesis does not imply that net photosynthesis rate of infested leaves will increase as spider mites suck out the whole cell content leading to local chlorotic spots. Possibly, the upregulation of the genes occurs because of the need to compensate for the loss of photosynthetic capacity within the area surrounding the chlorotic spots caused by the spider mites. Whether this keeping up appearances indeed coincides with a temporal increase in local photosynthesis remains to be clarified.

CsLOX genes respond to spider-mite infestation

CsLOX genes encoding lipoxygenases were among the strongest induced genes during very early infestation by spider mites, in both Cl and Co. Expression levels were already strongly induced at one day after infestation. Transcription of most *CsLOXs* decreased with progressing infestation. Interestingly, a few *CsLOX*s were down- instead of up-regulated during the infestation and for six others no transcripts were detected. Lipoxygenases are iron-containing enzymes that oxidize poly-unsaturated fatty acids and can be classified as 9- and 13-lipoxygenases, depending on the position of oxygen incorporation in their general substrates, linoleic acid and linolenic acid (Feussner and Wasternack, 2002). According to the sequence similarity, they can be classified into genes encoding Type I LOXs, enzymes which have no transit peptides (Feussner and Wasternack, 2002) and genes encoding Type II LOXs with putative chloroplast transit peptide sequence (Feussner and Wasternack, 2002). Characterized plant type-II LOX proteins all belong to the 13-LOX subfamily (Feussner and Wasternack, 2002) and catalyse formation of fatty-acid-derived short-chain volatiles (Chen et al., 2004, Mariutto et al., 2011) in plant defence responses. Type-I LOX proteins generally are 9-LOXs (Bannenberg et al., 2009), which have been shown to be involved in lateral root development and are responsive to pathogen infection (Gobel et al., 2002, Vellosillo et al.,

2007). Induction of *LOX* genes by herbivore infestation has been demonstrated in many plant species, including tomato (Fidantsef et al., 1999), tobacco (Voelckel et al., 2004) and rice (Duan et al., 2014). The *CsLOX*s form a gene family containing 23 members according to the first version of annotation of the cucumber genome (Huang et al., 2009, Liu et al., 2011). In this study, five more putative *CsLOX*s were identified based on the second version of the annotated cucumber genome. These 28 *CsLOX*s represent an expanded gene family compared to other species such as Arabidopsis (six members) and rice (15 members) (Umate, 2011). Nine cucumber *LOX* genes were identified as upregulated DEGs by infestation of spider mites in both accessions. Phylogenetic analysis of the *CsLOX*s showed there was no clear preference for induction of specific sub-clades by spider mites because similar numbers of type I and type II *CsLOXs* were up regulated. *CsLOX*s are also responsive to other abiotic or biotic stresses. Among the 23 previously identified *CsLOX*s, an increase in expression was found for 18 genes using qRT-PCR in response to at least one of the treatments including wounding of leaves, cold, salt, MeJA , JA, SA, ABA and powdery mildew (*Sphaerotheca fuliginea*) (Liu et al., 2011, Yang et al., 2012, Oh et al., 2014). Among the *LOXs* upregulated by spider-mite feeding, at least four were downregulated by downy mildew. A number of the spider-mite inducible *CsLOX*s were rapidly and transiently up-regulated already one day after infestation, and the relative expression of these genes remained high on the second and third day compared to non-infested plants. These rapid responses are in agreement with the rapid induction of GLV and accumulation of JA. Our results suggest that multiple *LOX* genes, both type I and type II, are involved in the defence reactions of cucumber towards spider mites. Elucidation of the specific biological role of each of these *LOX* genes in herbivore defence will need further study.

Genes involved in biosynthesis of terpenoids respond to spider mites

The changes in expression of genes involved in terpenoid biosynthesis suggest that this chemical class of compounds plays an important role in the response of cucumber to spider-mite infestation. Increased transcript accumulation was found for a gene encoding DXP-synthase, an important enzyme in the plastidic MEP/DOXP pathway. In contrast, a gene encoding HMG-CoA reductase was downregulated. This enzyme is in control of the rate of the mevalonate pathway, the other branch of terpenoid precursor biosynthesis in the cytosol. Two genes encoding the plastidic GPP synthase, the enzyme that catalyses the formation of the monoterpene substrate GPP, were also upregulated. Together this suggests that the biosynthetic flux of different parts of the terpenoid pathway is differentially regulated and that a shift occurs to the plastidic pathway at the expense of the cytosolic pathway. Moreover, spider-mite infestation resulted in transcript accumulation of several genes encoding terpene synthases, from un-detectable to relatively high levels. The two most induced *CsTPSs* are *Csa2M299880* and *Csa3M095040* of which no transcripts were detected in non-infested Cl and only very low levels in non-infested Co. Expression levels of

both *CsTPSs* increased with infestation time in both accessions. Furthermore, transcripts of seven genes encoding cytochrome P450 enzymes which were mapped in the GO / KEGG pathway of 'limonene and pinene degradation' were also differentially regulated during infestation by spider mites. Four of them were upregulated while three others were downregulated. However, whether these P450s are involved in terpenoid modification or breakdown remains to be determined. Spider-mite induced *TPS*s and altered volatile profiles were reported for several plant species including *C. sativus* (Kappers et al., 2010, Mercke et al., 2004). Quantitative and qualitative variation in emitted terpenoids was found in different cucumber accessions and coincides with different attractiveness to predatory mites (Kappers et al., 2010, Kappers et al., 2011). Changes in the expression of *CsTPS*s together with the genes functional in synthesis of terpene precursors and terpene modification will result in changes in the profile of terpenoids released by the plant and likely has consequences for the attractiveness towards predators.

Reconfiguration of cucurbitacin metabolism

To our surprise, genes associated with the biosynthesis of cucurbitacin C were down-regulated during the infestation by spider mites and this coincided with a decreasing trend in the content of cucurbitacin C during the first days of spider mite infestation. Cucurbitacins are triterpenoids that confer a bitter taste to cucurbits including cucumber (cucurbitacin C) and are known to discourage multiple herbivores such as beetles, larvae of lepidopteran species, cockroaches and spider mites (Agrawal et al., 2002, Balkema-Boomstra et al., 2003). Spider-mite performance in terms of egg deposition in our experiment was indeed better on accession Co, which is a non-bitter genotype. Introduction of spider mites on the cotyledons of bitter cucumber resulted in an increase in the cucurbitacin C concentration in the infested cotelydons and in the first systemic leaf seven to ten days after the spider mites were removed from the plants (Agrawal et al., 1999). However, in our study both cucurbitacin C concentration and biosynthesis associated genes (Shang et al., 2014) were downregulated during the first three days of infestation of cucumber. Although by unknown reason no transcripts were detected in our RNA-seq for the *Bi* gene [of which the *bi* allele /mutation is responsible for the loss of bitterness (Shang et al., 2014, Da Costa and Jones, 1971)], we found that transcript levels of the other eight genes in the cucurbitacin C pathway were reduced after introduction of spider mites in both accession Cl with bitter leaves (*Bi* allele) and accession Co with non-bitter leaves (*bi* allele). Furthermore, qPCR for the *Bi* gene itself in Cl showed a decrease in expression too. At first sight these results appear to be in conflict with those reported by Agrawal et al. (2002) although the different time frames in both studies could be of importance. In the experiments by Agrawal et al. (2002), the spider mites were introduced to cotyledons for three days and then removed using a miticide. Seven to ten days after that, the leaves (local and systemic) were collected and the concentration of cucurbitacin C measured. In our study, the spider mites were placed on the true leaves and

samples were collected on one, two or three days after infestation. It is possible that the expression of genes involved in cucurbitacin C biosynthesis and cucurbitacin C content only decreased locally, which we analysed, while they increase systemically, in organs more important to protect (e.g., younger leaves). However, in tomato it was demonstrated that different *T. urticae* lines differ in triggering plant JA defence (Kant et al., 2008). What is more, *Tetranychus evansi* can manipulate tomato plant defence by reducing the formation of induced defence compounds such as proteinase inhibitors while herbivory of *T. urticae* did induce these defence responses (Sarmento et al., 2011). Whether *T. urticae* indeed specifically represses direct defence in cucumber and/or whether this is specific for the mite strain used in our work, needs more in-depth study.

A gene (*Csa1M051740*) encoding a bHLH transcription factor was found to be co-expressed with the majority of cucurbitacin C biosynthesis associated genes in cucumber when analysing gene expression data upon infestation by spider mites and downy mildew (Adhikari et al., 2012) and data of tissue specific gene expression in unchallenged conditions (Li et al., 2011). It has been reported that *Bi* is regulated by two tandem *bHLH* genes. *Bl* (*Bitter leaf*, *Csa5G156220*), regulating *Bi* in leaves, and *Bt* (*Bitter fruit*, *Csa5G157230*), regulating *Bi* in fruits, respectively (Shang et al., 2014). However, in our experiments these two genes had low expression levels during the period of infestation with spider mites and their expression trends did not correlate with the changes in cucurbitacin C content. The presence of the seven E-box motifs in the promoter region of *Bi* suggests these bHLH transcription factors can affect *Bi* expression (Toledo-Ortiz et al., 2003) and hence this transcription factor is potentially regulating the expression of *Bi* cluster genes.

Conclusion

In summary, we analysed the transcriptional response in *C. sativus* to the two-spotted spider mite *T. urticae*. The study demonstrated that genes involved in JA signalling and the *LOX* gene family are significantly affected by herbivory by spider mites and cucumber plants try to keep up appearances by upregulating photosynthesis-related genes early during infestation. During herbivory of spider mites, genes associated with biosynthesis of volatile terpenoid compounds which are involved in indirect defence are upregulated while genes associated with biosynthesis of triterpene compound cucurbitacin C which is involved in direct defence are suppressed. These transcriptional changes match with the increased emission of terpenoids volatiles and decreased content of cucurbitacin C in cucumber leaves.

Material and Methods

Plant material and spider mite infestation

Cucumber (*Cucumis sativus*) accessions with bitter (Chinese long, 9930) or non-bitter foliage (Corona) were used for experiments. Seeds were germinated on previously autoclaved (80 °C for 6 hrs) peat soil (Lentse Potgrond®, No. 4,Ø 10 cm pots) and plants were cultivated in a greenhouse compartment with a 16 h day (22 ± 2 °C) and 8 h night period (18 ± 2 °C). The relative humidity was maintained at 60 to 70 %. All plants were watered every other day and were kept without chemical control against pests and diseases. The two-spotted spider mite *Tetranychus urticae* was reared on Lima bean (*Phaseolus vulgaris*) plants for many generations.

To compare spider-mite performance on both cucumber accessions used, female mites that originate from eggs deposited on a single day, and thus of the same age, were transferred to the abaxial side of leaf discs placed on water agar (0.5%) in a Petri dish. Leaf discs were incubated in low light at 20 ± 1 °C for 7 days and the number of eggs was counted. For each accession we used 10 leaf discs with 2 female mites per disc. The experiment was repeated three times.

For the RNA-seq experiment, plants were infested with 50 adult spider mites placed on the abaxial surface of the first fully expanded leaf of four-week-old plants using a fine brush. Leaves were collected at different time points after the onset of infestation, ranging from 15 minutes to 3 days and immediately flash frozen in liquid nitrogen, and stored at -80 °C until use. Control, non-infested leaves were sampled at the start of the experiment.

To estimate damage inflicted by the mites, pictures were taken by a Canon Power shot SX120 IS digital camera just prior to harvest. Pictures were analysed to estimate the damaged area using image J (Schneider et al., 2012). Hereto, 5 random spots of each 3 X 3 cm were selected and areas that were damaged (lighter in green intensity) were indicated by hand in image J. The damage was calculated as percentage of the total leaf area.

RNA isolation and qPCR

Frozen leaf samples were ground to a fine powder in liquid nitrogen using mortar and pestle and RNA was isolated using TriPure (Roche, Mannheim, Germany) and purified using the RNeasy Mini Kit (Qiagen, USA). Total RNA was treated using DNase I (New England BioLabs, Ipswich, MA, USA) for 30 minutes at 37°C. One µg of high quality [values of 260/280 and 260/230 were above 1.8 when measured with Nanodrop 2000 (Thermo scientific, Wilmington, USA)] DNA-free RNA was used for reverse transcription using the iScript cDNA syntheses kit (BioRad). Gene specific primers were designed for *C. sativus* genes based on

sequences obtained by BLAST search in the cucumber genome database (http://cmb.bnu.edu.cn/Cucumis_sativus_v2.0/ w). Primer sequences are shown in Table S4. Primers were tested for gene specificity by performing melt curve analysis. Quantitative RT-PCR analysis was done in optical 96-well plates with a MyIQ Single-Color Real-Time PCR Detection System (BioRad), using SYBR Green to monitor dsDNA synthesis. Each reaction contained 10 µl 2x SYBR Green Supermix Reagent (BioRad), 10 ng 10-fold diluted cDNA, and 300 nM of each gene-specific primer in a final volume of 20 µl. All qRT-PCR analyses were performed in biological and technical triplicates. The following PCR program was used for all PCR reactions: 3 min at 95 °C; 40 cycles of 10 sec at 95 °C and 45 sec at 47.5 °C. Threshold cycle (Ct) values were calculated using Optical System software, version 2.0 for MyIQ (BioRad). Subsequently, Ct values were normalized for differences in cDNA synthesis by subtracting the Ct value of a reference gene *Actin* (*Csa6M484600*) from the Ct value of the gene of interest. Normalized gene expression was then calculated as $2^{-\Delta\Delta Ct}$. Normalized gene expression values were used to calculate $\log_2$-transformed expression ratios for each experimental condition.

Library preparing and sequencing

Total RNA was extracted and purified as described above. mRNA was enriched using oligo (dT) magnetic beads and fragmented into about 200bp. Using the mRNA fragments as templates the first strand cDNA was synthesized by using random hexamer primers and reverse transcriptase (Invitrogen, USA). The second strand was synthesized by adding dNTPs, RNase H (Invitrogen, USA) and DNA polymerase I (New England BioLabs, USA). The double strand cDNA was purified with QiaQuick PCR extraction kit (Qiagen, Hilden, Germany) and washed with elution buffer of the kit to repair the end and add single nucleotide A (adenine). Then sequencing adaptors were ligated to the fragments. Fragments with required length were purified by agrose gel electrophoresis and enriched by PCR amplification. The library was then sequenced by Illumina HiSeq™ (Illumina, San Diego CA, USA).

RNA-seq data analysis

Quality control and mapping to the reference genome

Raw data (reads) were first processed using Perl (https://www.perl.org/) scripts to remove reads containing adapter, reads containing more than 10% unknown bases and low-quality reads. GC content and sequence duplication level of the clean data were calculated. Sequence saturation was measured by calculate the correlation of reads number and detected genes. All the downstream analyses were based on the clean data with high quality.

These reads were mapped to the public cucumber genome and annotation data base of accession 'Chinese long 9930' using SOAPaligner/soap2 (http://soap.genomics.org.cn/soapaligner.html).

Quantification of gene expression level

The Reads Per Kilobase of transcript per Million mapped reads (RPKM) method (Mortazavi et al., 2008) was used to calculate gene expression levels using the following formula: RPKM (X)=10^9 C/NL, where RPKM (X) is the expression of gene X; C is the number of mapped reads that is unique to gene X; N is the total number of mapable reads unique to all genes; and L is the number of bases on Gene X. The longest transcript of a given gene was used to calculate the expression level if there were more than one single transcripts for that gene.

Gene differential expression analysis

Differentially expressed genes were identified by comparison of sequenced libraries from different time points after infestation with those of non-infested plants for both accession Cl and accession Co. FDR (False Discovery Rate) of 0.01 and Log_2 (Treated/Control) fold-change >1 or <-1 were set as the thresholds to screen for differentially expressed genes.

Cluster analysis of differentially expressed genes

Differentially expressed genes were submitted to cluster analysis using Genemath (http://www.applied-maths.com). RPKM expression values were log_2 transformed and mean-centred with scaling of standard deviation of expression of each gene during the infestation of spider mites. Pearson correlation was used to calculate the pairwise distance and UPGMA (no weighted Pair Group Method with Arithmetic Mean) was used to summarize the distance between clusters.

GO function and pathway enrichment analysis of cucumber genes

All the detected cucumber genes in our samples were mapped to GO terms in the general Gene Ontology database (http://www.geneontology.org) as well as the pathway-related data base KEGG(Kanehisa et al., 2008). Gene numbers were calculated for every GO term and enrichments were analysed by agriGO (Du et al., 2010). The hypergeometric test was used to find significant enrichments and Hochberg [False Discovery Rate, FDR, Reiner et al. (2003)] method was used limit the false significance . Significance level was set as FDR corrected $P<0.05$. Pathway enrichments were performed using hypergeometric test (Evangelou et al., 2012). Threshold for significance was set as $p<0.05$.

Transcript profiling data from this paper will be deposited online.

Cucumber volatile collection and analysis

To investigate the induced volatile emission of cucumber, headspace samples of spider-mite infested cucumber plants were collected and analysed as previously described (Kappers et al., 2010). Spider-mite infested cucumber plants were individually placed in a closed glass jar and connected to two Tenax liners (20/35-mesh, Alltech). To collect volatiles, air was drawn through the jar with a flow rate of 150 ml min^{-1} over a Tenax liner that functions as inlet air filter. Volatile compounds released by the cucumber plant were trapped on the second, outlet tenax liner. Tenax liners with collected volatiles were dry-purged using a stream of nitrogen and subsequently analysed on a thermo-desorption GC (Thermo Trace GC Ultra, Thermo Fisher Scientific, Waltham, MA, USA) connected to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, MA, USA) quadrupole mass spectrometer as described in Kappers et al., (2010). Individual compounds were semi-quantified by calculation of the peak area under the curve and identified by comparison of mass spectra to those of authentic standards.

Jasmonic acid and salicylic acid measurements

Approximately 50 mg of ground leaf material was extracted in 1 ml 10% MeOH supplemented with 10^{-6} mol l^{-1} [$^2H_4$]-SA and [$^2H_6$]-JA used as internal standards. Samples were sonicated for 1 min at room temperature and subsequently shaked for 25 min at 4 °C at low frequency using a benchtop laboratory rotator. Thereafter, samples were centrifuged for 5 min at 4°C and 14000 rpm and the supernatant was pipetted into a new Eppendorf. The pellet was re-extracted twice with 1 ml of extraction solution and 1 min of vortexing. The combined supernatants were purified using Solid Phase Extraction columns (Stratax, Phenomenex, 30 mg/1cc) that were conditioned with 100% MeOH and water prior to use. After loading the sample, columns were washed with 10% MeOH and eluted using 80% MeOH (3 x 1 ml). Samples were evaporated to dryness by a gentile flow of N2 and stored at −20°C until analysis. Before LC-MS analysis, samples were reconstructed in 25 µl acetonitrile: 10 mM HCOOH (15:85, v:v) and analysed by a Acquity UPLC® system (Waters, Milford, MA, USA) together with triple quadrupole mass spectrometer XevoTM TQ MS (Waters MS Techonlogies, Manchester, UK). The conditioning procedure of the whole system was based on the protocol of (Balcke et al., 2012). Using MRM channels 211>151 and 211>133 for JA and 139>121 and 139>65 for SA. The MassLynxTM 4.1 software package was used for data acquiring and processing. For each time point, triplicate samples were analysed

Cucurbitacin C analysis using LC-MS

Leaf samples were ground into a fine powder in liquid nitrogen, and 100 mg fresh weight of frozen leaf powder was weighed and extracted with 300 mL of MeOH containing 0.1% (w/v) formic acid. Samples were briefly vortexed, sonicated for 30 min, and centrifuged at 21,000g for 10 min. Supernatants were filtered through a 0.2-mm inorganic membrane filter and transferred to HPLC vials for analysis. LC-MS profiling of crude leaf extracts was performed using an Water Synapt QToF MS based on the previously described method (De Vos et al., 2007). The generated mass chromatograms were processed (peak picking and baseline correction) in an unbiased manner using the MetAlign software package (Lommen, 2009), whereas metabolite reconstruction was performed using MSClust (Tikunov et al, 2012). Information on the relative peak height of the representative mass [M+H]+ 560.33491851 for cucurbitacin C was extracted at retention time previously recorded for a pure standard and the Knapsack database (http://kanaya.naist.jp/knapsack_jsp).

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Supplementary data

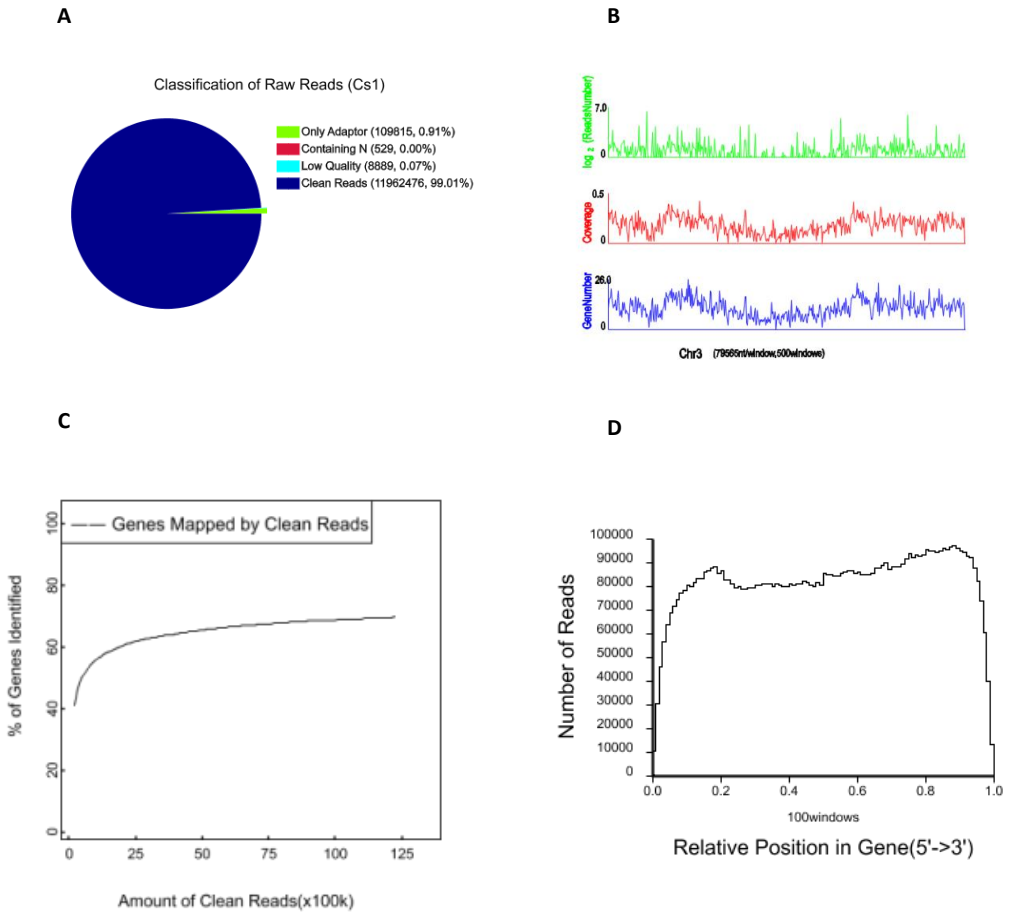


Fig. S1. Sequencing assessment of reads achieved from RNA-seq. (**A)** Number of raw reads classified by sequencing quality. (**B**) Distribution of reads mapped to cucumber chromosome 3. (**C)** Mapped reads and number of genes expressed. (**D)** Mapped reads and their relative position in genes.

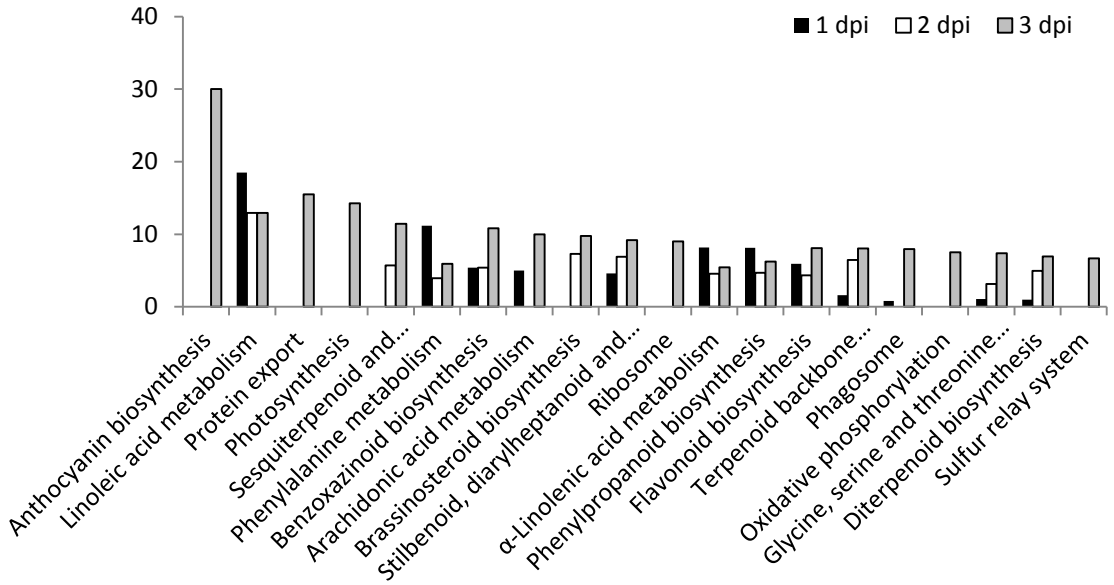


Fig. S2: Enriched KEGG pathways for spider-mite infested Chinese long cucumber leaves**.** KEGG pathway enrichment analysis was performed using hypergeometric test [Rivals et al. (2007), corrected $P \leq 0.05$]. For each KEGG pathway, the bars show DEGs as percentage of all genes annotated to that pathway after one, two or three days of spider-mite infestation.

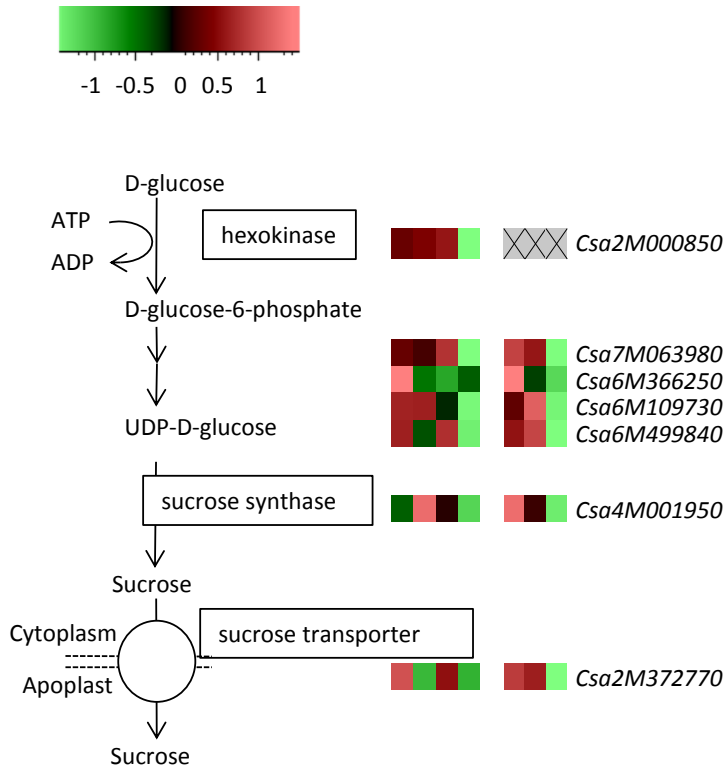


Fig. S3. The expression of sucrose-associated genes in cucumber during infestation of spider mites. Colour coding green represents relative low expression level and red represents high expression level to average expression of each gene during the infestation.

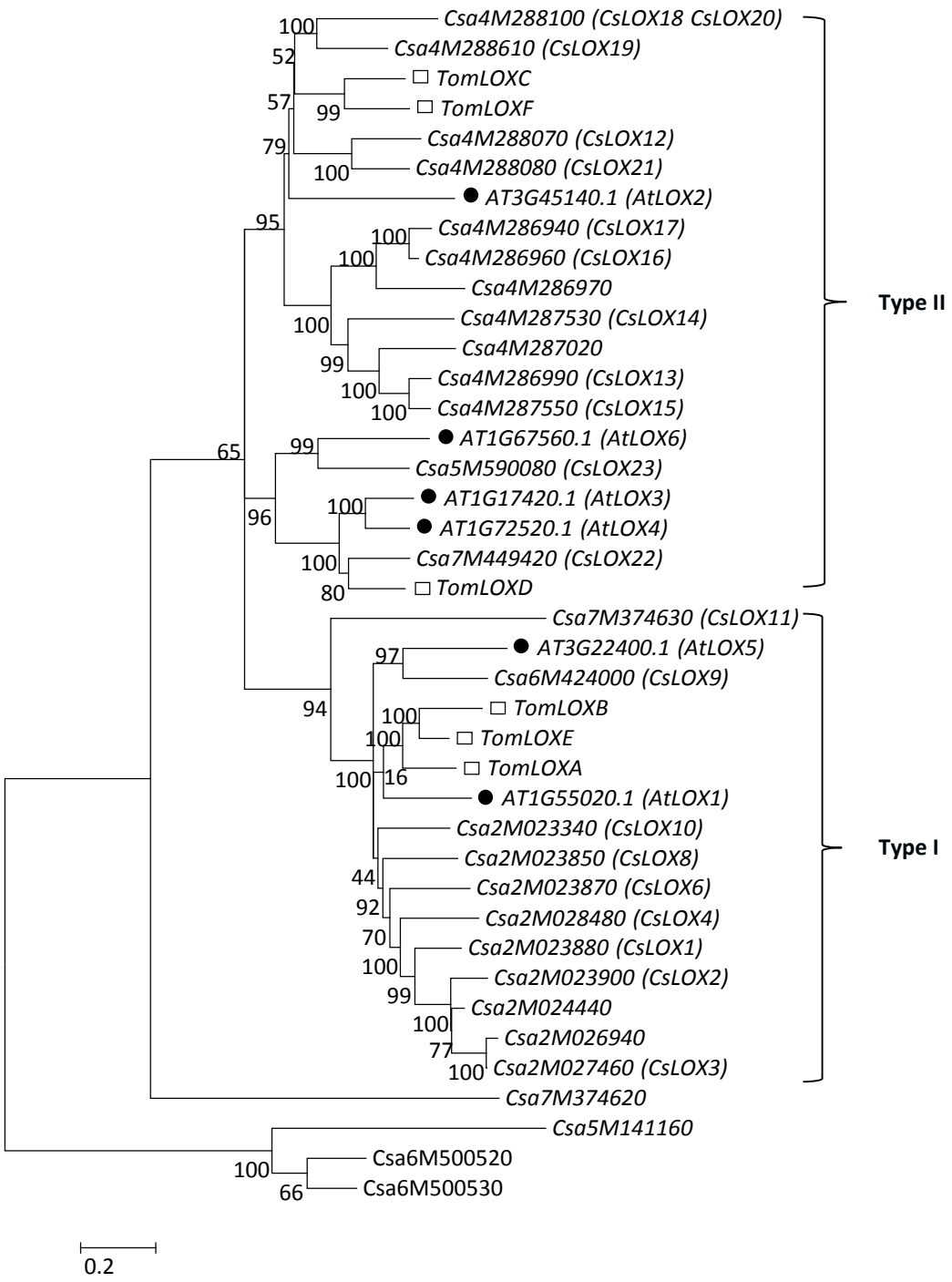


Fig. S4. Phylogeny analysis of cucumber putative type I and type II *LOX* genes with Arabidopsis *LOX*s (● *AtLOX*s) and tomato *LOX*s (□ *ClLOX*s)

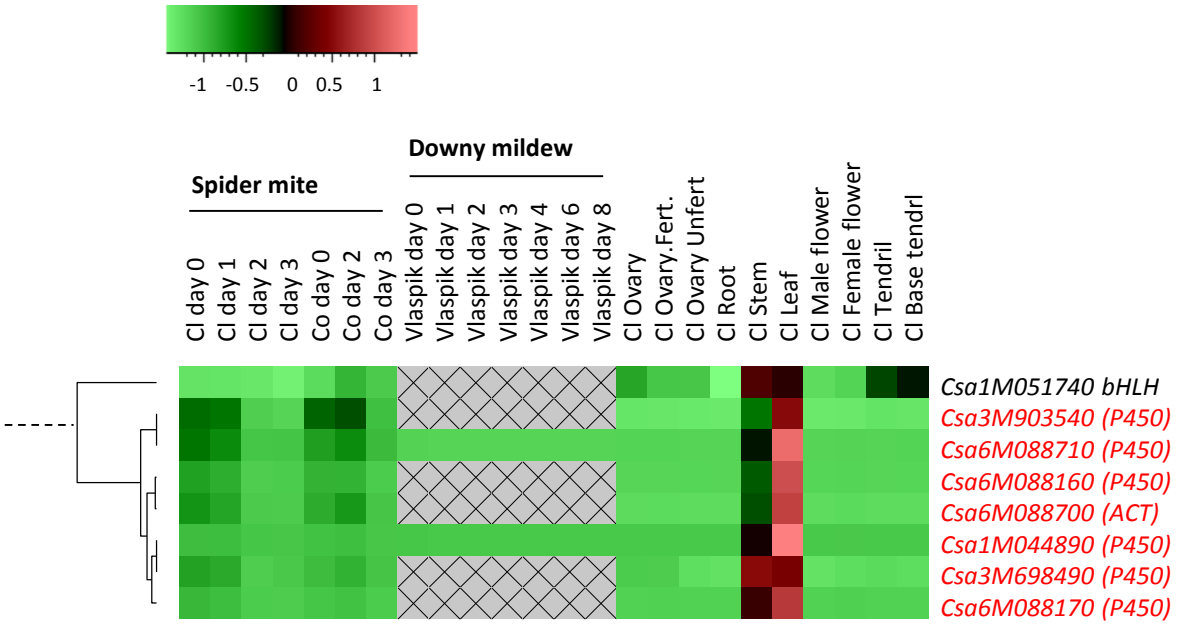


Fig. S5. Transcription factor co-expressed with *Bi* (*Bitter*) and related genes. All the TFs in the collection of DEGs were clustered with the genes involved in biosynthesis of cucurbitacin C. The TF most closely correlated with *Bi* and related genes are shown. Colour coding green represents relative low expression level and red represents high expression level.

Chapter 3

The role of transcription factors in the response to two-spotted spider-mite herbivory in cucumber

Jun He, Harro J. Bouwmeester, Marcel Dicke, Iris F. Kappers

Abstract

To gain insight into the regulatory networks that underlie the induced defence in cucumber against spider mites, genes encoding transcription factors (TFs) were identified in the cucumber (*Cucumis sativus*) genome and their regulation by two-spotted spider mite (*Tetranychus urticae*) herbivory analysed using RNA-seq. Of the total 1212 annotated TF genes in the cucumber genome, 119 were differentially regulated upon spider-mite herbivory during a period of only 3 days. These TF genes belong to different categories but the *MYB*, *bHLH*, *AP2/ERF* and *WRKY* TF gene families had the highest relative number of differentially expressed genes. A comparison with publicly available data on TF genes regulated by downy mildew (*Pseudoperonospora cubensis*) showed similar as well as different responses in various TF gene families. Correlation analysis of the expression of TF genes with metabolism-associated genes resulted in the putative identification of regulators of herbivore-induced terpenoid and green-leaf volatile biosynthesis. Analysis of the *cis*-acting regulatory elements (CAREs) present in the promoter regions of the genes responsive to spider-mite infestation revealed an over-representation of binding motifs for TFs such as MYBs, bHLHs and ERF/AP2s. This study describes the TF genes in cucumber that are potentially involved in the regulation of induced defence against herbivory by spider mites.

Keywords: cucumber, spider mite, transcription factor, promoter, *cis*-acting regulatory elements

Introduction

Transcription factors (TFs) are DNA-binding proteins that recognize and bind to specific regulatory sequences, the *cis*-acting regulatory elements (CAREs, also called CREs for *cis*-regulatory elements), in the promoter of the target genes (Latchman, 1997, Wittkopp and Kalay, 2012). In this way TFs can regulate gene expression by either activating or repressing transcription (Karin, 1990). CAREs are typically located adjacent to the promoter region of a gene, but can also be found in the gene itself, in introns or even further away from the gene, upstream or downstream from the promoter (Wittkopp and Kalay, 2012). By binding to these elements, TFs can stabilize or block the binding of RNA polymerase to DNA (Gill, 2001), catalyse the acetylation or deacetylation of histone proteins (Narlikar et al., 2002) or recruit co-activator or co-repressor proteins to form TF DNA complexes (Xu et al., 1999). All these processes can result in a change in the expression of the target gene of the corresponding TF.

TFs have been studied in the available plant genome sequences. For example, about 8-9% of the 27,411 protein-encoding genes in the Arabidopsis genome (TAIR10) were identified as putative TFs (Guo et al., 2005, Lehti-Shiu and Shiu, 2012). In the rice genome, about 10% of the predicted 22,896 genes (MSU Rice Genome Annotation, Release 7) were suggested to encode TFs and in tomato, about 6% of the genes in the genome putatively encode TFs (Sato et al., 2012). In cucumber, 1575 putative TFs were identified in the genome in the iTAK database (Lehti-Shiu and Shiu, 2012), which represents about 7% of the 23,248 predicted genes [(Huang et al., 2009, Li et al., 2011); version 2.0].

TFs play a role in the response of plants to various environmental stresses including the attack by herbivores. Plants perceive this attack via damage- or herbivore-associated molecular patterns [DAMPS or HAMPS, Mithofer and Boland (2008)]. Upon recognition of the attack, plant hormones moderate the response to the different attackers, although the exact mechanisms are unclear (Lazebnik et al., 2014). Subsequently, the hormone signalling is translated into the activation or repression of gene expression. This transcriptional regulation usually depends on the transcription and translation of TFs (Wu and Baldwin, 2010), although other factors such as chromatin-remodelling could also be involved (Berr et al., 2012). The transcriptional changes of for example metabolism-associated genes, finally results in an altered metabolic profile of the infested plant (Wu and Baldwin, 2010).

Transcription factors can be classified into different families according to the conserved DNA-binding domains and over sixty families of TFs have been identified in various plant species (Perez-Rodriguez et al., 2010). Well-known TF families are MYB, bHLH, ERF, NAC, bZIP, WRKY, MADS, HSF, TCP, GATA, NF-Y, ZF-TF, ARF, HOX and SBP. Among them, particularly the MYB, bHLH, WRKY, AP2/ERF, NAC and bZIP families have been shown to play a role in plant defence (Seo and Choi, 2015).

The MYB TFs form a large, functionally diverse superfamily. They are characterized by the

conserved MYB domain which generally comprises up to four imperfect helix-turn-helix structure repeats (Paz-Ares et al., 1987). Up to 198 MYB TFs were identified in the Arabidopsis genome (Chen et al., 2006) and 252 in soybean (Du et al., 2012). MYBs are involved in the response of plants to abiotic stress, such as drought (Urao et al., 1993) and biotic stress, such as feeding of aphids (Liu et al., 2010). In Arabidopsis, *AtMYB51* (*HIG1*, *HIGH INDOLIC GLUCOSINOLATE 1*) expression was increased by mechanical stimuli and this in turn up-regulated the expression of genes involved in the biosynthesis of indolic glucosinolates (Gigolashvili et al., 2007), a class of plant secondary metabolites that function as anti-herbivore compounds (Kim and Jander, 2007, Muller et al., 2010). *AtMYB75* has been found to play a role in the regulation of anthocyanin biosynthesis (Borevitz et al., 2000) and the flavonol pathway (Tohge et al., 2005, Yonekura-Sakakibara et al., 2007, Bhargava et al., 2010). Overexpression of *AtMYB75* increased both anthocyanin and flavonol levels in transgenic Arabidopsis and enhanced resistance to generalist caterpillars (Onkokesung et al., 2014). However, the same plants became more susceptible to specialist caterpillars (Onkokesung et al., 2014). MYBs involved in the regulation of the expression of genes involved in metabolism and inducible defences were also identified in other species such as *Populus tremuloides* (Mellway et al., 2009) and *Nicotiana attenuata* (Kaur et al., 2010).

bHLH (basic helix-loop-helix) TFs have a conserved bHLH domain which comprises about 60 amino acids, including a basic region for DNA binding and a helix-loop-helix region for interaction with proteins (Ferredamare et al., 1994). In the genome of Arabidopsis, rice and poplar, 170, 178 and 99 *bHLHs* were identified, respectively (Carretero-Paulet et al., 2010). Functions of bHLH TFs include regulation of hormone signalling (Abe et al., 1997, Lee et al., 2006), flavonoid biosynthesis (Li, 2014) and stress responses (dePater et al., 1997, Kiribuchi et al., 2004). AtMYC2, AtMYC3 and AtMYC4 were shown to be involved in the jasmonic acid (JA) signalling pathway (Lorenzo et al., 2004, Fernandez-Calvo et al., 2011) and defence to *Helicoverpa armigera* (Dombrecht et al., 2007) and *Spodoptera littoralis* (Schweizer et al., 2013). Three JA-associated MYC2-like (AtJAM1, 2, 3) proteins were identified as targets of AtMYC2 and negatively regulate other targets of AtMYC2 including genes involved in anthocyanin biosynthesis and defence against herbivores in Arabidopsis (Sasaki-Sekimoto et al., 2013). In *N. attenuata,* NaMYC2 plays a role in the regulation of nicotine and phenolamide biosynthesis when these plants are infested by insect herbivores (Woldemariam et al., 2013).

The ERF (AP2/ERF, APETALA2/ethylene-responsive element binding factor) family are TFs containing one or two AP2 domains. The AP2/ERF family has been studied in many species including Arabidopsis (147 genes) and rice (174 genes), (Nakano et al., 2006). ERF TFs could be subdivided into different groups including AP2, ERF and RAV. The ERF group contains DREBs (dehydration-responsive element-binding proteins) and ERFs. DREBs can bind to C-repeat/dehydration-responsive elements (DRE/CRT, CCGAC) and ERFs can bind to GCC boxes (GCCGCC) in promoter regions. Many ERF TFs are essential regulators of plant responses to biotic and abiotic stress. Arabidopsis AtERF5, AtERF6 and AtRAP2.2 have been implicated in

resistance to *Botrytis cinerea* mediated by JA and ethylene (Moffat et al., 2012, Zhao et al., 2012). AtERF6 is the substrate for mitogen-activated protein kinases (MAPKs), MAPK3 and MAPK6, and can be phosphorylated by the two MAPKs (Meng et al., 2013). Phosphorylated AtERF6 is more stable *in vivo* and is able to activate JA-responsive defensive genes including *PDF1.1* and *PDF1.2* independent of ethylene. In rice, OsERF3 with an EAR (ERF-associated amphiphilic repression) motif is essential for resistance to the chewing striped stem borer *Chilo suppressalis* via transcriptional regulation of two MAPKs and two WRKY TFs, mediating JA and salicylic acid (SA) signalling and the formation of trypsin protease inhibitors (TrypPIs) (Lu et al., 2011). In contrast, *OsERF3* transcription was suppressed by the piercing and sucking brown planthopper *Nilaparvata lugens* (Lu et al., 2011).

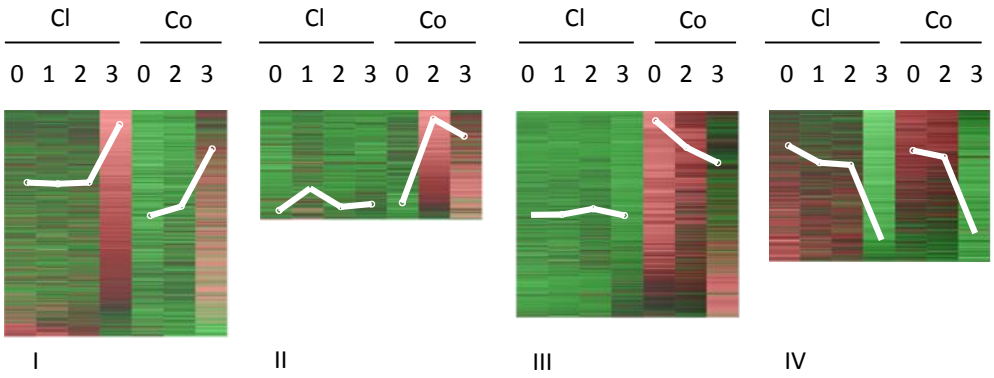
The WRKY TF family is a class of transcription factors that all have a domain of about 60 amino acids that includes the WRKYGQK motif at the N-terminal and a zinc-finger-like motif at the C-terminal (Rushton et al., 1995). The WRKYGQK motif binds to the W-box in the promoter regions of its target genes (Rushton et al., 1996). There are at least 75 WRKY family members in *Arabidopsis* (Wu et al., 2005) and 109 in rice (Eulgem and Somssich, 2007, Wu et al., 2005, Ross et al., 2007). WRKY TFs are of major importance in plant responses to various biotic stresses including bacterial (Deslandes et al., 2002) and fungal (Zheng et al., 2006) infection. WRKYs also play a role in induced resistance to herbivores. *N. attenuata* WRKY3 (NaWRKY3) and WRKY6 (NaWRKY6) co-ordinately regulate JA-involved defence to *Manduca sexta* (Skibbe et al., 2008). In rice, OsWRKY70 is a positive key regulator of JA-mediated defences but a negative regulator of gibberellin-mediated growth (Li et al., 2015). Overexpression of *OsWRKY70* increased defence to chewing herbivore *C. suppressalis* but enhanced susceptibility to piercing herbivore *N. lugens* (Li et al., 2015).

In this study, we studied the involvement of TFs and CAREs in cucumber in the response to the two-spotted spider mite (*Tetranychus urticae*). Cucumber is an important vegetable grown and consumed world-wide (http://faostat.fao.org). The chelicerate two-spotted spider mite is a major pest, which feeds on plants in more than 140 plant families (Van Leeuwen et al., 2010) including important agricultural crops such as maize, grape, tomato, pepper and cucumber. Global transcriptional changes in response to infestation by spider mites were demonstrated to occur in Arabidopsis (Zhurov et al., 2014) and tomato (Martel et al., 2015) with 841 and 2,133 differentially expressed genes (DEGs), respectively, which included hundreds of TF genes. For example, 187 TF genes, belonging to multiple families, were present among the tomato DEGs (Martel et al., 2015). Previously, we have analysed the transcriptional changes in cucumber during the first three days of spider-mite infestation and identified more than 2000 DEGs (Chapter 2). Here, we focus on the regulation of the expression of TF genes by spider-mite herbivory and include a comparison with the response upon infection with a pathogenic fungus. Furthermore, we analysed promoter sequences of genes that are affected in their expression due to infestation by spider mites for possible TF binding sites.

Results

Cucumber genes responsive to spider mites

Previously, using an RNA-seq approach we identified 2348 genes that were differentially expressed between spider mite infested and non-infested plants of two genotypes of cucumber, accession Chinese long with bitter and accession Corona with non-bitter foliage (Chapter 2). All DEGs were organized into four groups based on their expression at different time points after infestation (Fig. 1). In group I, gene expression increased during the period of infestation in both accessions whereas group IV contains genes of which expression decreased in both accessions. Groups II and III include those genes of which transcripts remained more or less unaltered in Chinese long but increased (group II) or decreased (group III) in Corona after mite infestation. Statistical analysis showed that both duration of infestation and the genotype had significant effects on the transcript abundance of genes and there was a significant interaction between both factors in three of the four groups of genes (Fig. 1). We used this collection of DEGs as data set to identify TFs that play a role in both positive and negative early responses towards two-spotted spider mites in cucumber. We compared our results with the transcriptional response of cucumber towards the pathogen *Pseudoperonospora cubensis* downy mildew as described by Adhikari et al. (2012) to study similarities and differences in the involvement of TFs in the response of cucumber to these two different biotic stresses.



	I	II	III	IV
Accession	*P*=0.028	*P*<0.001	*P*<0.001	*P*=0.026
Day	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001
Accession x Day	*P*=0.004	*P*<0.001	*P*<0.001	*P*=0.192

Fig. 1. Regulation of gene expression by infestation of spider mites during one, two or three days in two cucumber accession [Chinese long (Cl) and Corona (Co), upper part] and effects of accessions and days of infestation on the expression of the DEGs [(*P*-value), lower part]. All DEGs were self-organized using GeneMaths XT (http://www.applied-maths.com) into four groups (I to IV) based on their expression at different time points of infestation. White lines indicate the general expression trend for each group. DEGs from each group were tested separately using a general linear model (GLM) (IBM SPSS STATISTICS, version 22). Up-regulated or down-regulated abundance of transcripts was set as variable and the accessions and days of infestation by spider mites were set as fixed factors. The effects of the factors and the combined effects of two factors were tested.

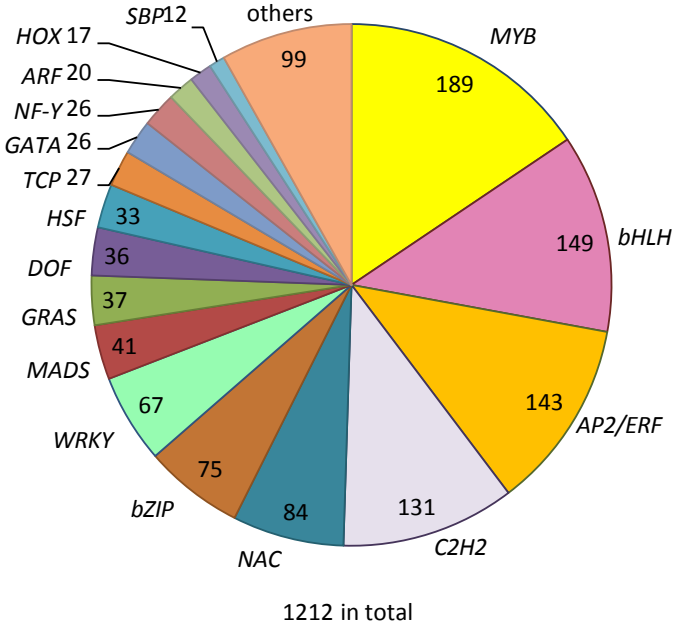


Fig. 2. Transcription factor gene families identified in cucumber with numbers indicating number of TF genes belonging to a particular family.

Regulation of TF expression in response to spider-mite herbivory

Based on the annotated set of genes predicted from the cucumber genome (http://www.icugi.org/cgi-bin/ICuGI/index.cgi), 1212 genes with a putative TF function of different classes were retrieved (Fig. 2). Half of all TF genes belong to only four different TF gene families, i.e. *MYB* (189), *bHLH* (149), *AP2/ERF* (143) and *C2H2* zinc fingers (131). Furthermore, among the 1212 TF genes there are 84 genes encoding NAC domain proteins, 73 genes encoding bZIP (basic-leucine zipper domain) proteins and 67 genes encoding WRKYs. In addition, there are 10 TF gene families with less than 50 members each, including *MADS* (41 genes), *GRAS* (37 genes), *WTHT* (33 genes), *TCP* (27 genes), *GATA* (26 genes), *NF-Y* (26 genes), *ZF-TF* (22 genes), *ARF* (20 genes), *HOX* (17 genes) and 99 putative TFs that do not belong to any of the above TF families. Out of these 1212, transcription of 119 TF genes was differentially regulated by spider-mite infestation, of which 60 were generally upregulated (in group I and group II DEGs) and 59 were downregulated (in groups III and IV). Among these 119 TF genes, the *WRKY* family was relatively most represented as 21% of the *WRKY* genes putatively present in the cucumber genome were differentially expressed upon spider-mite infestation, followed by *AP2/ERF* (16%), *bHLH* (14%) and *MYB* (10%) TF genes (Table 1). The distribution of the number of genes of each TF gene family in the different groups of DEGs was analysed (Table 1) to discover general trends in the expression of these genes. Group I and group II, both representing increased gene transcripts after mite infestation, included 5 *MYB*s. In contrast, 7 *MYB*s were present in group III and 2 in group IV, which both represent genes of which transcription is repressed by spider-mite feeding. TF genes of the *bHLH* family

were almost equally distributed over the four groups, with 4 to 6 genes in each of the groups. Most of the *ERF* TF genes were included in group I (4) and group II (13) and displayed increased transcription upon spider-mite infestation, while 7 *ERF* genes displayed decreasing transcription, 6 in group III and 1 in group IV. Most of the *WRKY* genes were assigned to group III or group IV, and thus displayed decreasing transcription in response to spider-mite infestation. Considering the general trends of the expression of genes in each group of DEGs, it seems *ERF*s were preferentially upregulated and *WRKY*s preferentially downregulated upon spider-mite herbivory, while *MYB*s and *bHLH*s were about equally up- and downregulated. For the other TF families, fewer genes were found to be regulated by spider-mite infestation and they were randomly distributed over the 4 groups of DEGs (Table. 2).

Table 1. Number (Nr) of TF genes belonging to different classes/families that are differentially regulated by spider mites and downy mildew.

	Spider mites						**Downy mildew**		**Spider mites and downy mildew**	
	Group I	**Group II**	**Group III**	**Group IV**	**In Total**	**%***	**Nr**	**%**	**Nr**	**%**
MYB	5	5	7	2	19	10%	26	14%	5	3%
bHLH	6	5	6	4	21	14%	22	15%	6	4%
AP2/ERF	4	13	6	1	24	16%	29	20%	14	10%
C2H2	1	4	4	1	10	8%	18	14%	2	2%
NAC	1	2	3	1	7	8%	28	33%	4	5%
bZIP	1	0	2	0	3	4%	15	21%	0	0%
WRKY	2	3	7	2	14	21%	31	46%	12	18%
MADS	0	0	1	0	1	2%	4	10%	0	0%
GRAS	0	0	3	0	3	8%	10	27%	1	3%
DOF	0	0	3	0	3	8%	4	11%	0	0%
HSF	0	0	1	0	1	3%	7	21%	0	0%
TCP	1	0	0	0	1	4%	5	19%	0	0%
GATA	1	0	0	0	1	4%	5	19%	0	0%
NF-Y	0	0	1	1	2	8%	5	19%	0	0%
ARF	1	0	0	0	1	10%	3	15%	0	0%
HOX	1	0	1	0	2	12%	0	0%	0	0%
SBP	0	0	0	0	0	0%	0	0%	0	0%
others	4	0	1	1	6	6%	18	18%	2	2%
In Total	28	32	46	13	119	10%	241	19%	46	4%

*percentage of genes per category that is affected by the indicated stress.

As the *WRKY*, *AP2/ERF, bHLH* and *MYB* TF genes were relatively most responsive to spider mites and are also reported to encode important TFs in plant defence (Seo and Choi, 2015), we made a more detailed analysis of their expression pattern during early spider-mite infestation (Fig. 3). Expression profiles of *MYB*s and *bHLH*s during the first three days after the onset of mite infestation were found equally up- and downregulated (Fig. 3), logically corresponding to their classification in the DEG self-organizing maps (Fig. 1). Most of the *AP2/ERF*s were found to be upregulated after mite infestation, particularly in Corona. The majority of *WRKY*s were found in group III and thus were suppressed by the infestation. However, Fig. 3 shows that transcripts of most of *WRKY*s actually increased on the first or second day of mite infestation before transcripts were down-regulated on the third day of infestation.

To understand whether TFs affected by spider-mite herbivory also are involved in the responses of cucumber to pathogen infection, we used a published data set (Adhikari et al., 2012) in which transcriptional changes in cucumber (accession "Vlaspik") upon downy mildew infestation were profiled using RNA-seq. Downy mildew infection for different periods ranging from one to eight days resulted in 230 putative TFs genes of different classes that were differently expressed compared to non-infected leaves: 26 *MYB*, 22 *bHLH*, 30 *ERF*, 18 *C2C2*, 28 *NAC*,15 *bZIP*, 31 *WRKY*, 4 *MADS*, 10 *GRAS*, 7 *WTHT*, 5 *TCP*, 5 *GATA*, 5 *NF-Y*, 3 *ARF*, and 18 other TFs (Adhikari et al., 2012) (Table 2). *WRKY, AP2/ERF*, *bHLH*, and *MYB* genes were among the TF gene families that were relatively most responsive towards spider mite infestation, while *WRKY*, *NAC* and *GRAS* were among the strongest affected TF genes upon downy mildew infestation (Table 1). In general, downy mildew infection resulted in more TF gene families with DEGs than spider mite infestation. By comparing both datasets we identified 46 TF genes that were differentially expressed as a result of downy-mildew infection as well as spider-mite infestation, although not always in the same direction (see below). Hierarchical cluster analysis showed that these genes clustered into three groups (Fig. 4). The first group consists of 14 TF genes of which transcripts were downregulated by downy-mildew infection but upregulated in response to spider-mite infestation, including two *NAC*s, two *MYB*s, two *bHLH*, three *WRKY*s and five *ERF*s. The second group contains 13 TF genes upregulated by both downy mildew and spider mites and including a *NAC*, two *bHLH*s, four *WRKY*s and six *ERF*s. The third group included 16 TF genes that were mainly upregulated by downy mildew but less or even downregulated by spider mites. This group included a *TFIIA*, a *NAC*, a *GRAS*, two *bHLH*s, three *MYB*s, three *ERF*s and five *WRKY*s.

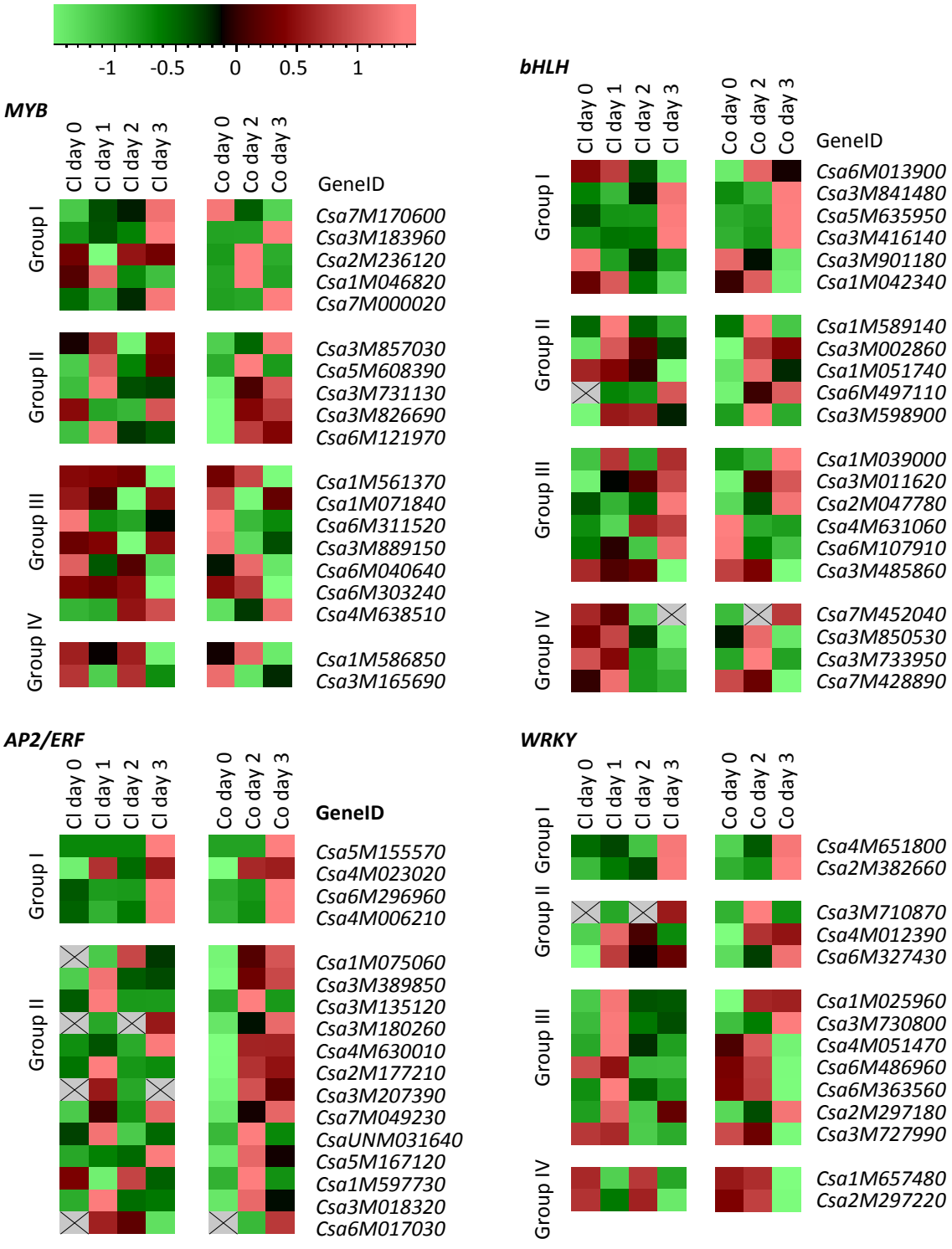


Fig. 3. Expression profiling of cucumber transcription factors of four families most differentially regulated by infestation by spider mites. RPKM values of the genes obtained from RNA-seq were introduced into Genemaths XT and were normalized to the standard deviation of each gene during the infestation. Colour coding: green represents low expression; red represents high expression. Grey squares with cross indicate that no transcripts were detected

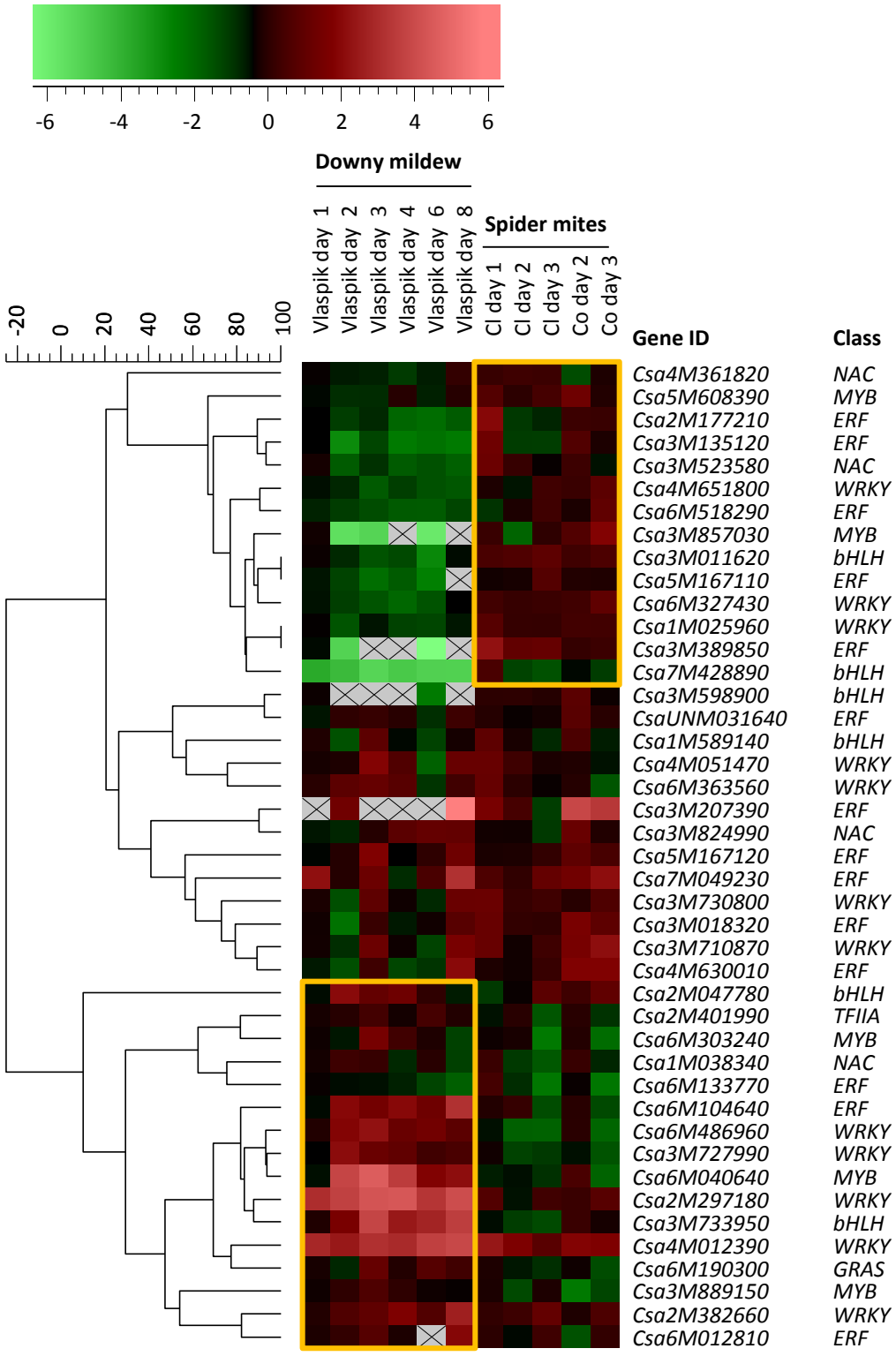


Fig. 4. Hierarchical clustering analysis of TFs regulated by spider-mite feeding and downy-mildew infection according to the expression profile over different time points in each experiment. Expression was normalized as $\log_2$ (day n/day 0), n= 1, 2 or 3 for the spider-mite experiment and 1, 2, 3, 4, 6 and 8 for the downy-mildew data set extracted from a published dataset (Adhikari et al., 2012). Colour coding: green represents low expression; red represents high expression. Grey areas with cross indicate that no transcripts were detected. Yellow rectangles indicate blocks of genes that have particularly high similarity in expression.

Strongest influenced TF genes by spider-mite infestation

Spider-mite infestation resulted in differentially expressed transcripts of TF genes in both directions. The relative transcript abundance of a putative *bHLH* gene (*Csa3M002860*) increased about 20-fold (log_2Ration≈4.4) one day after the onset of mite infestation compared to transcription in non-infested plants and this gene was the strongest affected TF-gene found in spider-mite infested cucumber leaves. Although the absolute abundance of the transcripts of this TF gene decreased on days 2 and 3, expression was still about 14- and 10-fold higher than in non-infested plants, respectively (Fig. 5). A similar pattern was found for a *MYB* TF gene, *Csa6M121970*, with the strongest induction (18-fold) on day 1, and about 7- and 6-fold higher compared with uninfected on succeeding days. The other putative TF genes that are depicted in the right panel of Fig. 5 have strongest induction of expression on different days, but all belong to the ten most upregulated TFs. This group includes three *ERF*s. In contrast, the two relatively strongest down-regulated TF genes belong to the *bHLH* family (Fig. 5, left panel). Transcription of the *bHLH* TF gene, *Csa3M850530*, first increased slightly after one day of infestation and subsequently decreased to approximately half the amount of transcripts of non-infested leaves on day 2 and to 8% (log_2Ration≈3.6) on day 3. Expression of the *bHLH* TF gene, *Csa7M452040*, showed a similar pattern, resulting in a decrease of transcripts to 10% (log_2Ratio≈3.3) compared to non-infested plants after three days. The expression of the ten strongest suppressed TF genes decreased mainly on day 3 and this category includes 3 *MYB*s.

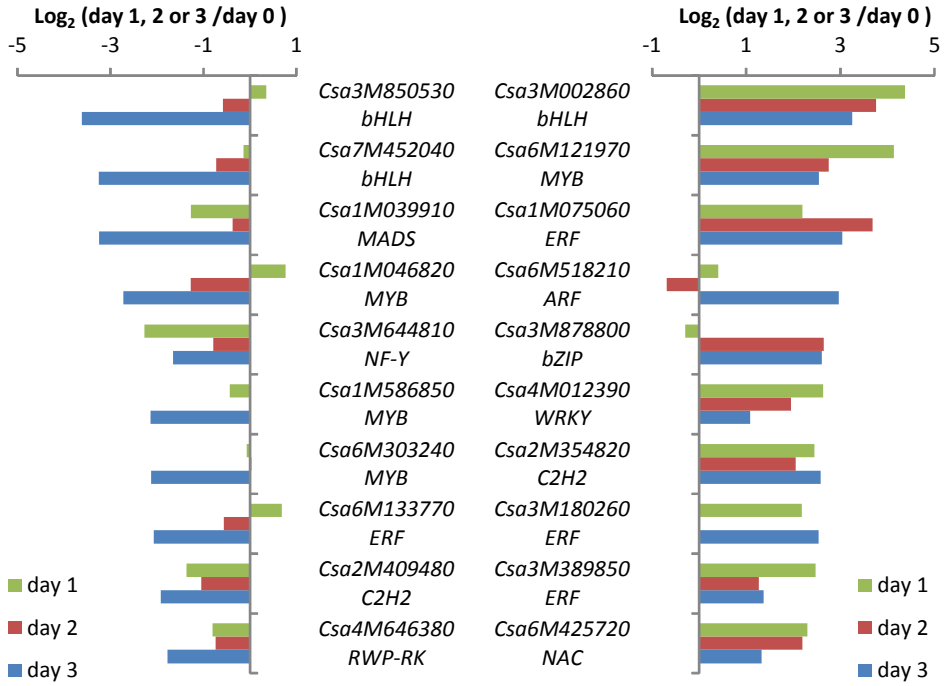


Fig. 5. Top 10 strongest down- and up-regulated transcription factor genes in cucumber accession Chinese long after one, two or three days of spider-mite infestation.

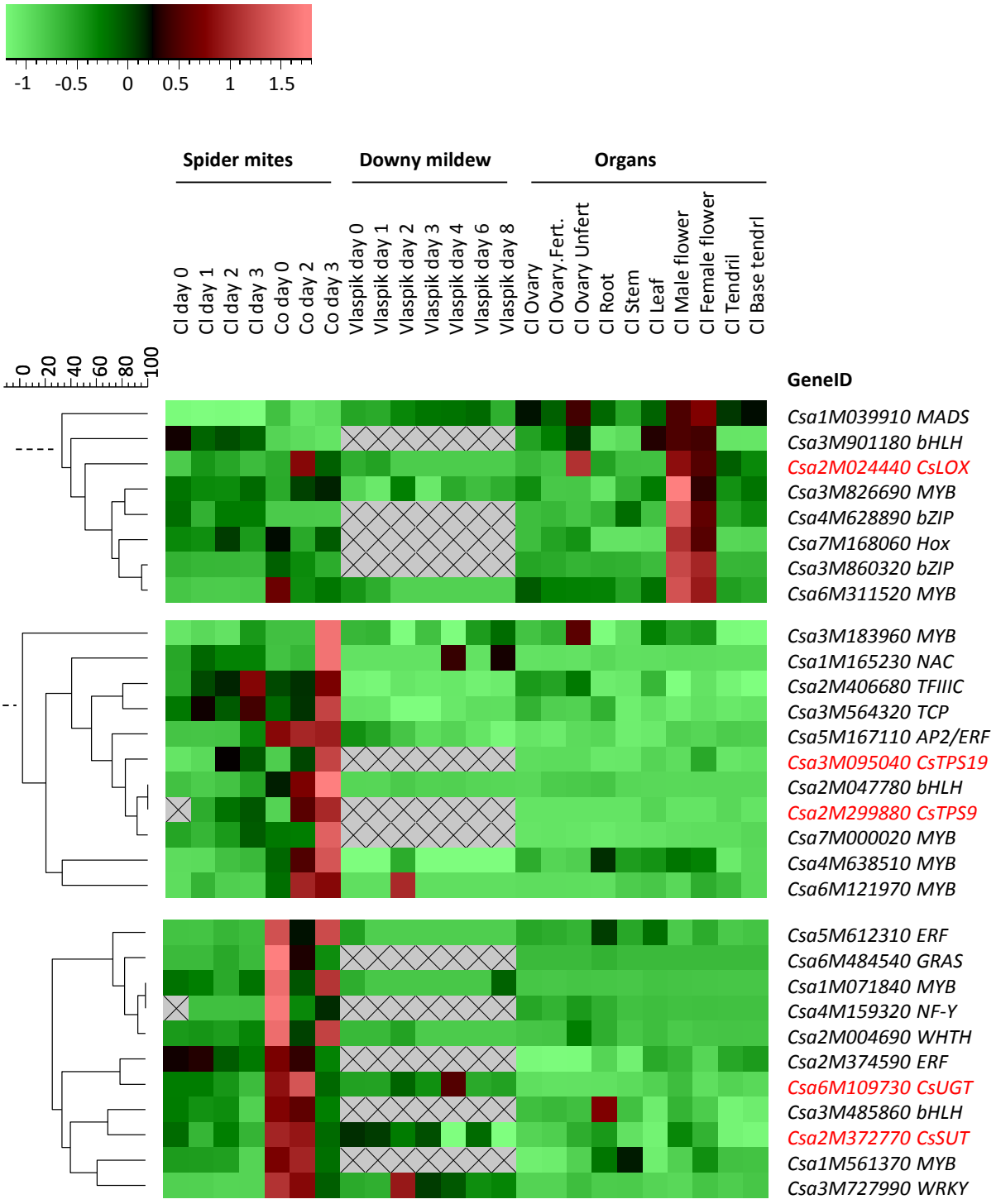


Fig. 6. Co-expression of a selection of metabolism-associated genes (*CsLOX*, *CsTPS9*, Cs*TPS19,* Cs*UGT,* Cs*SUT*) and TF genes during infestation by spider mites, downy mildew (Adhikari et al., 2012) infection and in different – non-infested/non-infected – organs (Li et al., 2011) of cucumber accessions Chinese long (Cl), Corona (Co) and Vlaspik. Colour coding: green represents low expression and red represents high expression. Grey areas with cross indicate that no transcripts were detected..

Co-expression of TF genes with defence related genes

Co-expression analysis between TF genes and genes that encode proteins associated with biosynthesis of defence-related metabolites may give insight in their regulatory relationships. As we have particularly interest in genes and pathways that are involved in biosynthesis of compounds within the herbivory-induced volatile blend that is of importance for attraction of natural enemies of spider mites, we focused on the expression profiles of a gene encoding a lipoxygenase (LOX*)* which may be involved in green leaf volatile (Gigot et al., 2010, Mercke et al., 2004) and/or JA biosynthesis (Wasternack and Hause, 2013) and two genes encoding terpene synthases that synthesize multiple mono- and sesquiterpenes and were found to be induced upon spider-mite infestation (Chapters 2 and 4). Furthermore, we included a gene encoding an UDP-glycosyltransferase (UGT), *CsUGT,* putatively involved in the glycosylation of secondary metabolites (King et al., 2000) and a gene encoding a sucrose transporter (SUT), *CsSUT,* both of which were selected as representatives of the genes downregulated by spider-mite infestation. In addition to the gene expression patterns detected in response to spider-mite infestation, expression profiles of these genes after infection with downy mildew (Adhikari et al., 2012) and in different organs of unchallenged plants (Li et al., 2011) were included to analyse co-expression relationships with the 119 selected TF genes that show differential expression upon spider-mite herbivory. Hierarchical clustering analysis showed that the TF genes closest co-expressed with *LOX* (*P-value*=0.05) are a *HOX*, two *bZIPs* and two *MYBs* (Fig. 6). Both spider-mite induced *TPS*s closely co-expressed with each other and with a *bHLH*, a *MYB* and an *ERF* TF (*P-value*=0.04). Furthermore, althought not significantly, three other *MYB*s, a *NAC*, a *TFIIIC* and a *TCP*, co-expressed with both *TPS* genes more than other TF genes (*P-value*=0.08). *CsUGT* and *CsSUT* co-expressed with an *ERF*, a *bHLH,* a *MYB* and a *WRKY* (*P-value*=0.05) (Fig. 6).

In addition, the strongest induced TF, i.e. *MYC* (*Csa3M002860*) co-expressed with a gene encoding DXS (1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE), and 8 other genes that encode proteins with unknown function (Table S2). All these genes were upregulated during the infestation by spider mites, especially in accession Corona. Seven genes co-expressed with the strongest suppressed TF *bHLH* (*Csa3M002860*), however protein functions of these genes are mostly unknown except for one gene that encodes a putative lyase.

Various motifs were enriched in the promoters of genes responsive to spider mites

The 2 kb upstream sequences of all 2348 spider-mite induced DEGs were analysed for *cis*-acting regulatory elements (CAREs) by alignment to a dataset of TF binding motifs in Arabidopsis (Franco-Zorrilla et al., 2014). Compared to randomly shuffled sequences, multiple motifs were found enriched in the promoters of these differentially expressed (either up- or downregulated) genes (Fig. 1). For example, the MYB55_2 element which can

bind to MYB TFs, the MYC2, MYC3, and MYC4 elements (all binding sites of MYC TFs) and DEAR3 (binding site of ERF TFs) were the five most enriched motifs found in the 2 kb promoter sequence of both upregulated and downregulated genes (Table 2). Furthermore, binding motifs PIF4, PIF5, PIF3 that can bind to bHLH TF, MYB binding motifs MYB46_2 and MYB111_2, AHL binding motif AHL12, ERF/AP2 binding motifs ATERF1, DEAR4_2, ORA47_2, bZIP binding motif bZIP60_2 were found to be enriched in the promoters of both up- and downregulated genes. No motifs were identified that were specifically and significantly enriched in either the group of up- or downregulated promoter sequences.

Table 2. The top15 putative TF binding motifs enriched in the 2 kb promoter regions of upregulated and downregulated genes compared to random sequences.

Sequence Logo	Name	Adjusted *P*-value		Binding TF
		In upregulated	In downregulated	
	MYB55_2	1.91E-53	1.13E-43	MYB
	MYC3	4.86E-53	5.13E-45	bHLH
	MYC4	7.68E-47	1.49E-40	bHLH
	MYC2	4.07E-43	4.29E-35	bHLH
	DEAR3	1.50E-42	1.94E-32	ERF/AP2
	PIF4	8.88E-42	1.67E-31	bHLH
	PIF5	1.78E-41	3.55E-32	bHLH
	PIF3	3.28E-32	3.40E-24	bHLH,
	MYB46_2	6.84E-31	8.84E-29	MYB
	AHL12_3ary	9.34E-29	8.20E-17	AHL
	ATERF1	2.57E-28	2.45E-18	ERF/AP2
	bZIP60_2	1.11E-25	9.88E-16	bZIP
	DEAR4_2	1.20E-25	6.20E-17	ERF/AP2
	MYB111_2	3.30E-25	6.92E-24	MYB
	ORA47_2	1.08E-23	1.02E-15	AP2/ERF

Note: Enrichment analysis was performed using AME (http://meme.ebi.edu.au/meme-4.10.1/tools/ame) by aligning to the database of DNA-binding motifs of transcription factors in Arabidopsis. Enrichment was determined relative to randomly shuffled input sequences. Wilcoxon rank-sum test was used to calculate the *P*-value for significance of the enrichment, and Bonferroni correction was used to adjust it for multiple tests (adjusted *P*-value).

Motifs in promoters of defence-related genes

The binding motifs on the promoter regions of spider-mite inducible *CsLOX*, *CsTPS9* and spider-mite suppressed *CsUGT* and *CsSUT* were analysed to obtain more hints for possible regulators. Multiple motifs were found on each of the promoters by aligning the sequences to PlantCARE, the database of plant *cis*-acting regulatory elements (Lescot et al., 2002). The number of non-redundant elements found in the promoter of *CsLOX* was 32, there were 35 in the promoter of *CsTPS9*, and 24 and 30 in the promoters of *CsUGT* and *CsSUT,* respectively. Thirteen elements were present in all four promoters, including motifs HSE, circadian, Box I, G-box, TTC-rich repeats, CGTCA-motif, AAGAA-motif, TATA-box, CAAT-box, and 3 unnamed motifs (Fig. 7). The promoters of the upregulated genes, *CsLOX* and *CsTPS9*, shared an AE-box, an MBS and a motif with unknown function, while there was no common motif shared by the promoters of the downregulated *CsSUT* and *CsUGT*. Every one of these promoters also had multiple specific motifs which were not present in the other three.

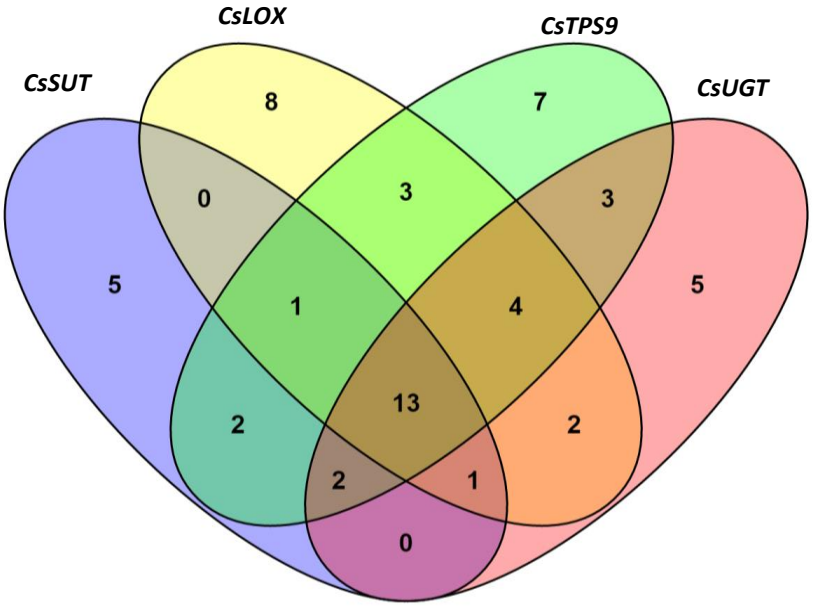


Fig. 7. Specific and shared binding motifs found in promoter sequences of *CsLOX* (upregulated), *CsTPS9* (upregulated), *CsUGT* (downregulated) and *CsSUT* (downregulated).

Discussion

A central goal in improving our understanding of the response of plants to biotic stresses is to identify genes that are responsive to the stress and determining how they are regulated and what their role is in the plant under the biotic stress of interest. With the development of genomic technologies applicable for more (crop) species, including methods for gene

expression profiling, these issues can be addressed on a more global scale. Here, we examined the transcriptional reprogramming of *C. sativus* TF genes in response to spider-mite herbivory. We identified 119 TF genes that are differentially expressed upon mite infestation and we compared expression profiles of these TF genes with the expression profiles that were reported upon infection with an oomycete pathogen.

TF genes in the cucumber genome

TFs are key regulators of plant gene expression and often connect (hormonal) signalling pathways to biosynthetic pathways. We used the RNA-seq dataset in which two *C. sativus* genotypes were compared for their transcriptional response upon two-spotted spider mite infestation (Chapter 2) to study the role of TFs in the response of cucumber to spider-mite herbivory. Identification of TF genes among the DEGs induced by spider-mite feeding may help to understand the complexity of the defence regulatory networks. In order to get an overall view on TF genes within the cucumber genome, we first identified all 1,212 putative TF genes among the 23,248 predicted cucumber genes according to the feature domains on the encoded proteins. This number is lower than the 1575 putative TF genes identified in the iTAK database (Lehti-Shiu and Shiu, 2012). However, annotation of the TF genes collected in our study is based on alignments to published genes in Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and therefore considered to be more reliable. Moreover, the number of differentially expressed TF genes belonging to major defence-regulated TF families such as *MYB*, *bHLH*, *AP/ERF*s, *NAC*, *WRKY* are comparable with the identification used in the present study or based on iTAK annotation. TF genes represent 5.2% (6.7% if iTAK database is used) of the protein encoding genes in the cucumber genome. This is comparable to the 5.7% of TF genes in tomato (Sato et al., 2012), but less than the 8.9% (based on iTAK) in Arabidopsis and 10.6% in rice (iTAK).

TF genes responsive to spider-mite infestation

Previously, we found that early spider-mite infestation at a relatively low infestation pressure resulted in about 10% of the genes being differentially expressed (Chapter 2). Likewise, little more than 10% of the putatively annotated TF genes significantly changed expression upon spider-mite infestation. About 21% of the genes encoding WKRYs in the cucumber genome were responsive to infestation by spider mites, making the WRKYs the most affected TF family. The relative contribution of responsive genes within the *AP2/ERF* and *bHLH* gene families was more than the average responsive level. Furthermore, *MYB* and *ARF* gene families were around or little above average response levels and this may imply that *WKRYs*, *AP2/ERFs* and *bHLHs* play more important roles than other TF gene families in

the regulation of defence processes to spider-mite feeding. *MYBs*, *bHLHs* and *ERFs* were also in the top three regulated TF gene families in tomato infested by spider mites for different periods ranging from one to 24 hours, resulting in 187 differentially expressed TF genes among the 2132 DEGs (Martel et al., 2015). In contrast to the relative contribution of spider-mite regulated *WRKY* TF genes in cucumber (14 out of 119 TF genes), only nine *WRKY*s (out of 187) were regulated in tomato in response to spider-mite infestation. Transcripts of six of the tomato *WRKYs* increased during the spider-mite herbivory and three decreased.

The expression patterns of different TF families

Based on expression changes, four TF families including *MYB*, *bHLH*, *AP2/ERF* and *WRKY* may play an important role in mediating transcriptional regulation in plant inducible defence in cucumber. The *MYBs* form a large family of TF genes and have diverse functions in growth, development and defence. Induction of the expression of *MYB* TF genes in response to herbivory was also observed in Arabidopsis (Gigolashvili et al., 2007), *Populus trichocarpa* (Mellway et al., 2009) and *N. attenuata* (Kaur et al., 2010). Multiple identified MYB TFs such as AtMYB51, AtMYB75, PpMYB134, NtMYBJS1 and NaMYB8 have been reported to affect biosynthesis of various defensive secondary metabolites by regulating the expression of associated biosynthesis genes (Gigolashvili et al., 2007, Borevitz et al., 2000, Mellway et al., 2009, Gális et al., 2006, Kaur et al., 2010). The nine TF genes that are co-expressed with *CsTPS9* and *CsTPS19*, during the infestation of spider mites, downy mildew and in different cucumber organs include four *MYBs*. Furthermore, two *MYBs* have an almost similar expression pattern as *CsLOX*, suggesting that these MYBs play an essential role in the regulation of (herbivore) induced volatile (terpenoids and green leaf volatiles) formation in cucumber.

The *bHLH* family in cucumber is smaller than the *MYB* family but relatively more genes in this family were responsive to spider mites. Like *MYB*s, differentially expressed *bHLH* TF genes included both up-regulated and down-regulated genes in response to spider mite infestation. Functional characterisation of AtMYC2 (with a bHLH domain) showed that it is able to up-regulate a group of genes and suppress another group of genes which both are involved in the JA signalling pathway (Lorenzo et al., 2004, Dombrecht et al., 2007). Moreover, a triple mutant of Arabidopsis lacking AtMYC2, AtMYC3, and AtMYC4 became extremely susceptible to the generalist herbivore *Spodoptera littoralis* (Schweizer et al., 2013). MYCs can interact with each other as well as with other proteins such as JAZ to control the regulation nodes of plant processes involved in induced defences (Fernandez-Calvo et al., 2011, Sasaki-Sekimoto et al., 2013). Considering that the cucumber response to spider mites also involves changes in the concentration of JA and changes in the expression of JA-regulated genes (Chapter 2), this suggests that the *bHLH*s identified among the DEGs may be involved in these processes. For example, the *bHLH* TF, *Csa2M047780*, which is co-expressed with *CsTPS9* and *CsTPS19*

may be involved in the regulation of these two genes, possibly through JA signalling.

The largest number of TF genes responsive to spider-mite feeding are *AP2/ERF* TF genes. Different from *MYB*s and *bHLH*s, most members of this group of *AP2/ERF*s had higher expression at least on one day post infestation compared to non-infested plants and only one gene was down-regulated. AP2/ERF TFs are essential regulators involved in the JA and ethylene signalling pathways. Constitutive expression of *AtERF5* or *AtERF6* in Arabidopsis resulted in significant upregulation of JA- and ethylene–responsive genes, downregulation of SA-mediated signalling and increased resistance to the fungal necrotroph *Botrytis cinerea* (Moffat et al., 2012). Infestation of Arabidopsis by the generalist *Spodoptera exigua* or specialist *Pieris rapae* caterpillars resulted in differential upregulation of many *AP2/ERF*s (Rehrig et al., 2014). In rice, *OsERF*3 is rapidly responsive to feeding of *C. suppressalis* and increases the expression of two mitogen-activated protein kinases and two *WRKY* genes, the concentrations of JA, SA and the activity of trypsin protease inhibitors (Lu et al., 2011). One cucumber *ERF* is among the nine TF genes co-expressed with *CsTPS9* and *CsTPS19*, implying a possible role for the corresponding protein in the regulation of the biosynthesis of terpenoid volatiles in cucumber in response to herbivory.

The contribution of induced TF genes within the *WRKY* family is higher than observed for other TF families. *WRKYs* have been reported to play a pivotal role in defence (Eulgem et al., 2000, Chen et al., 2012). The first day after the onset of infestation by spider mites, more *WRKY* genes were upregulated than downregulated, but progressive infestation resulted in decreased transcripts of most *WRKYs* that were responsive to mites. Co-expression analysis did not result in identification of possible metabolism related genes affected by these *WRKYs* except for *Cs3M27990* that was found to be co-expressed with negatively regulated Cs*UGT* and Cs*SUT* during infestation. The role of WRKYs in defence against plant pathogens is well documented (Pandey and Somssich, 2009) while little is known about their role in defence against herbivory. However, WRKY3 and WRKY6 co-ordinately regulate defence in *N. attenuata* to herbivores in a JA-dependent way. The identified cucumber *WRKY*s which were up-regulated by spider mites could potentially encode regulators for JA-involved defence against these herbivores.

Conserved TFs in response to biotic stresses

Overlap between DEGs in response to infestation by spider mites and downy mildew represent general responses of cucumber to herbivory and pathogen infection and more than half of the overlapping TF genes included *WRKY* and *AP2/ERF* genes, although various expression patterns were detected for responses to both biotic stresses. This again suggests an essential role of these two TF families in plant defence. Comparison of the expression patterns of the 48 TF genes regulated by infestation by both biotic stress agents illustrated

shared and divergent responses to the two biotic stresses. All the 14 shared *AP2/ERF* genes were up-regulated by spider mites in at least one of the time points post infestation compared to non-infested plants while six of them were found downregulated by downy mildew and others upregulated. The divergence of these expression patterns are expected to have consequences for the expression of the target genes of these TFs. However, the number of TF genes which were induced by both spider mites and downy mildew was higher than the number of TF genes which showed contradictory expression patterns during the two infestation processes. These commonly upregulated TF genes are most likely involved in a shared basal response of cucumber to biotic stress. Similar to this, considerable overlap in transcriptional changes was induced in Arabidopsis by infestation by the pathogenic leaf-infecting bacterium, *Pseudomonas syringae* pv. tomato, the pathogenic leaf-infecting fungus *Alternaria brassicicola*, the chewing caterpillar *Pieris rapae*, the cell-content feeding thrips *Frankliniella occidentalis* and the phloem-sucking aphid *Myzus persicae* (De Vos et al., 2005). We analysed whether plant species representing different families share TF genes that are induced upon spider-mite herbivory. Hereto, we searched for tomato and Arabidopsis orthologues of the *ERF*, *bHLH* and *WRKY* TF genes that were regulated in cucumber upon spider-mite infestation using published datasets (Martel et al., 2015, Zhurov et al., 2014). Although there are not many orthologues present in the overlapping DEGs, a number of orthologous genes between cucumber and tomato or cucumber and Arabidopsis were responsive to infestation by two-spotted spider mites (Fig. S1). Cucumber *Csa6M296960* and its orthologous gene in tomato, *Solyc09g007260.2.1*, both encode an *ERF* TF, and their transcripts were upregulated in cucumber, especially on day three after the onset of mite infestation, and after six hours in tomato. Three cucumber *bHLH*s regulated in response to spider-mite infestation have orthologues in tomato including two that were downregulated in both species while the third one was upregulated in both species upon spider-mite infestation. Two *WRKY*s were induced by spider-mite feeding after three days in cucumber, of which the tomato orthologues were also upregulated. Cucumber and Arabidopsis also share orthologous genes encoding an ERF TF and three encoding WRKY TFs. However, the orthologous *ERF* and two *WRKY*s were induced in cucumber but suppressed in Arabidopsis upon mite infestation. Another *WRKY* was suppressed in cucumber but induced in Arabidopsis. The range of periods of infestation on cucumber (from one to three days) was different from Arabidopsis and tomato (from one to 24 hours) making it difficult to compare the expression patterns of genes. However, there were a number of orthologous TF genes that are regulated the same in different plant species showing the conserved relevance of these TFs in the response to feeding by spider mites.

Possible regulatory relationships between TFs and metabolite biosynthesis associated genes

The analysis of the CAREs located on the promoters of the spider-mite induced DEGs

provides information to understand the regulation by TFs. Enrichment in the promoters of the DEGs of MYB, MYC2, MYC3, MYC4, PIF3, PIF4, PIF5, DEAR3, DEAR4_2, ATERF1, ORA47_2, RRTF1, REM1_2 and DREB2C motifs implies that DEGs as result of spider-mite infestation are very likely recognized and regulated by the MYB, bHLH and AP2/ERF TFs. Depending on the different functions of the TFs binding to them, these motifs could mediate the up- or down-regulation of the expression of the adjacent genes.

TFs are key regulators of plant defence. However, TF proteins cannot repel herbivores and the defence of the plant relies on morphological restructuring (such as changes in trichome density) or re-configuration of secondary metabolism. The genes involved in secondary metabolite biosynthesis in plants are likely regulated by TFs and the understanding of these regulatory relationships are core questions to understand mechanisms of plant defence. In cucumber, a number of essential genes that are involved in direct and indirect defence to spider mites have been identified including the pathway genes involved in biosynthesis of cucurbitacin C, which was shown to be regulated by two bHLH TFs in leaves and fruits, respectively (Shang et al., 2014) and *CsTPSs* involved in the biosynthesis of terpenoids that are of importance for the attraction of natural enemies of spider mites (Mercke et al., 2004, Kappers et al., 2010, Kappers et al., 2011). In this study we analysed the co-expression of these defence-related genes using gene expression data of plants exposed to spider-mite infestation (Chapter 2), infection with downy mildew (Adhikari et al., 2012) as well as of different organs of cucumber that were not exposed to biotic stress (Li et al., 2011). Although there will be a time delay between the abundance of TFs, the expression of their target genes and post translationally regulated processes, co-expression of biosynthetic genes with TF genes could indicate putative candidate key-regulators. *CsTPS9* and *CsTPS19* were hardly expressed in any of the organs of cucumber but induced in leaves upon spider-mite infestation and it is possible that TFs including *MYB*, *NAC*, *AP2/ERF* and *bHLH* which were only highly expressed in spider-mite infested leaves, play a role in the regulation of the expression of these genes. Twenty one motifs were found in the promoter sequences of the spider-mite induced *CsTPS9* and *CsTPS19* and 16 motifs were found in the promoters of both spider-mite suppressed genes *CsSUT* and *CsUGT*. In contrast, 13 motifs were present in all four promoters analysed indicating possible binding by similar types of TFs, implying that up- or down-regulation of a particular gene could be the result of binding to different members of each TF family or the interaction between different TF proteins, or between TFs and other co-regulators.

In conclusion, we have shown that TFs play an important role in the response of cucumber to infestaton by spider mites. *MYB*, *bHLH*, *AP2/ERF* and *WRKY* TF genes were among the most regulated TFs during the first days of infestation by spider mites. Further work to confirm the involvement of these TFs in the induced indirect defence of cucumber is in progress.

Material and methods

Grouping of the DEGs

Leaves of cucumber (*Cucumis sativus* accession Chinese Long (Cl, with bitter foliage) and Corona (Co, with non-bitter foliage) were infested with two-spotted spider mites (*Tetranychus urticae*) for different time periods and gene-wide gene expression was analysed using a RNA-seq approach leading to identification of differentially expressed genes (DEGs) as described in chapter 2 of this thesis. To create a self-organizing map (SOM), expression values of DEGs were imported into GeneMaths XT (http://www.applied-maths.com) and normalized to the standard deviation of all the measured points of each gene of different accessions and time points. Pearson correlation was used to compare the similarity of the expression patterns of the genes. The number of nodes in the X-dimension and Y-dimension are both set to 2. The effects of genotype and time point since infestation were tested using a general linear model (GLM) (IBM SPSS STATISTICS, version 22). RPKM (Reads Per Kilobase of transcript per Million mapped reads) values of the genes were set as dependent variables, accessions and days post infestation were set as fixed factors. The list of DEGs responsive to downy mildew and their expression information were obtained from published data (Adhikari et al., 2012). Expression data of cucumber organs and DEGs in tomato leaves infested by spider mites are from published datasets (Li et al., 2011, Martel et al., 2015).

Identification of TF genes in the cucumber genome and responses to infestation by spider mites

The genome sequence and predicted genes of *C. sativus* L. accession 9930 (Chinese Long) were downloaded from the Cucurbit Genomics Database [(Huang et al., 2009, Li et al., 2011); version 2.0] for DEG analysis and TF identification. To analyse the RNA-seq data for putative TFs, a literature search was done and the sequences were aligned to online databases including Genbank non-redundant protein sequences database and InterPro protein signature databases (http://www.ebi.ac.uk/interpro/). Genes encoding proteins with specific domains for different TF families were identified as TF genes. TFs in the collection of DEGs in response to infestation by spider mites were identified as responsive TF genes and were selected for further analysis. For comparison of cucumber TF transcriptional responses to spider mite infestation to such responses to downy mildew infection, the TF genes in the corresponding collection of DEGs were selected. A complete list of all TF gene names and abbreviations can be found in Table S1.

Expression patterns and co-expression analysis of TF genes and metabolite biosynthesis associated genes

The RPKM values of the selected genes were imported into Genemaths XT and expression levels were centralized and normalized to the standard deviation for each gene over the analysed time points. Different accessions as well as different infestations were analysed separately for expression patterns of selected genes. For co-expression of TFs with selected possible target genes, expression values of the genes from different accessions, different infestations and different organs (Li et al., 2011) were analysed together. The similarity of expressions of the genes were analysed by Pearson correlation and the genes were clustered using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method.

Enrichment analysis of motifs

The 2 kb promoter regions of the up-regulated and down-regulated genes were extracted from the published cucumber genome [Huang et al. (2009), version 2] into two groups separately. Motif enrichment in each group of promoters was performed using an online motif enrichment analysis service AME (http://meme.ebi.edu.au/meme-4.10.1/tools/ame) by aligning promoter sequences to the database of DNA-binding specificities of plant transcription factors in Arabidopsis (Franco-Zorrilla et al., 2014). AME identified motifs that are relatively enriched in nucleotide sequences were compared with shuffled (randomized) sequences. Wilcoxon rank-sum test was used for calculating the *P*-value, and Bonferroni correction was used to adjust it for multiple tests (adjusted *P*-value). The selected promoter sequences were searched using an online service PlantCARE [plant *cis*-acting regulatory element, Lescot et al. (2002)].

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Supplementary data

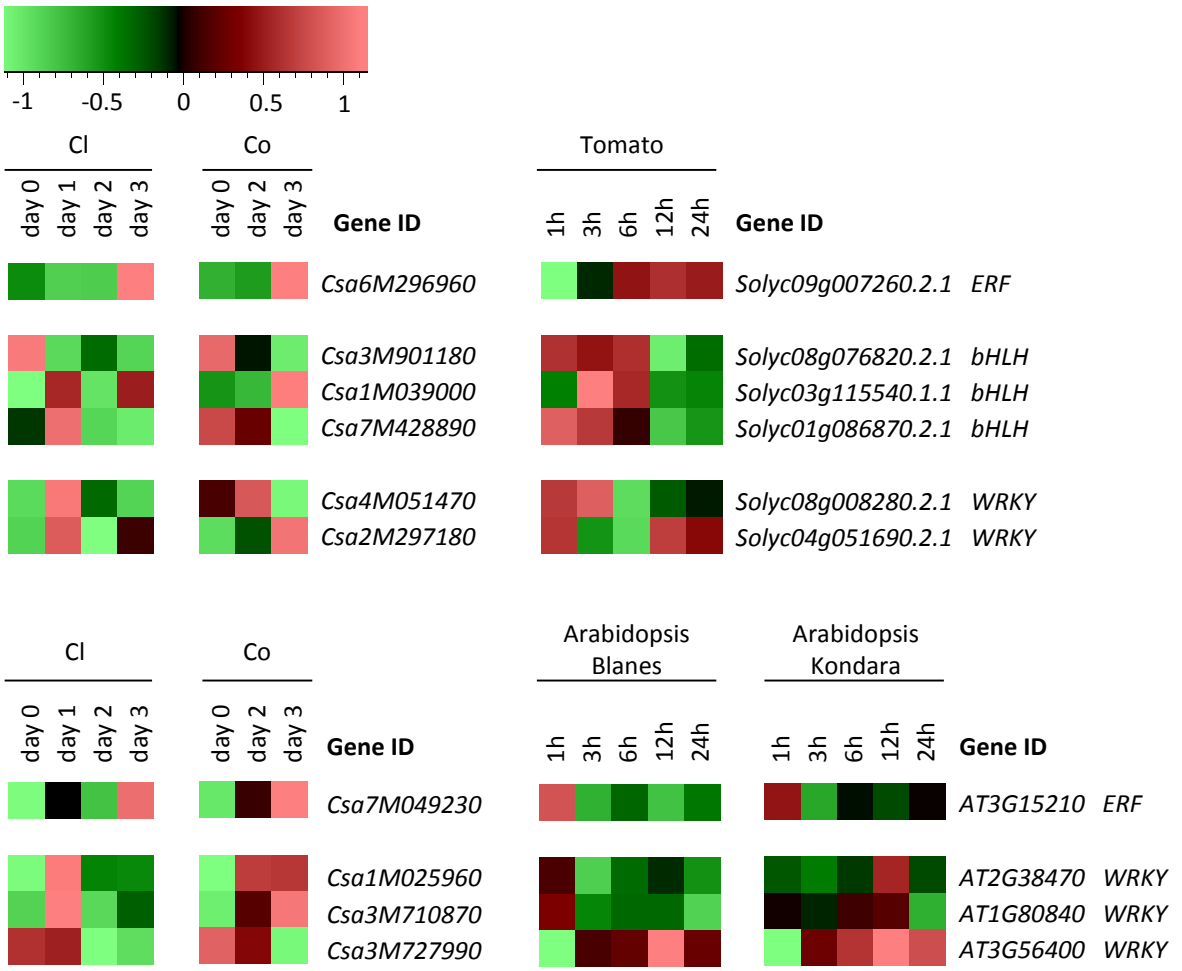

Fig. S1. *ERF, bHLH* and *WRKY* orthologues present in both cucumber and tomato (upper part) or cucumber and Arabidopsis (lower part) regulated by spider-mite infestation. Colour coding: green represents low expression and red represents high expression.

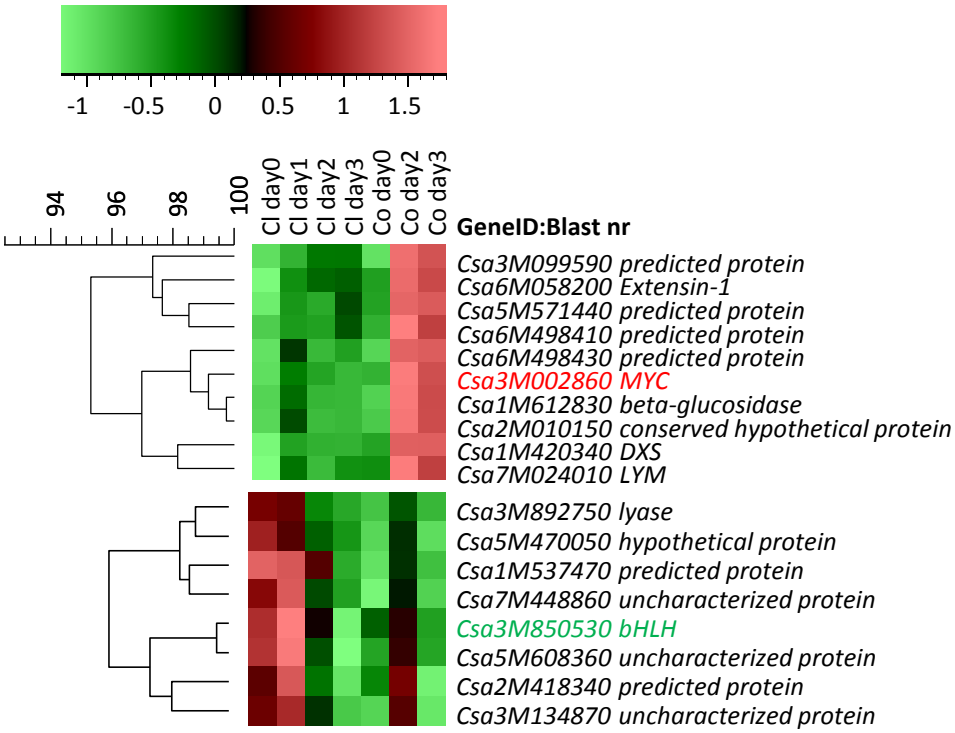

Fig. S2. Differentially expressed genes in cucumber regulated by spider mites which were closest co-expressed with the strongest induced (upper part) or supressed (lower part) transcription factor during the first three days of infestation.

Chapter 4

Characterization of the terpene synthase family in cucumber

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Submitted

Abstract

Terpenoids are a class of compounds that play multiple roles in plants. They play important roles in a.o. the flavour, pollinator attraction and defence of plants. In cucumber (*Cucumis sativus*) they have been reported to be important components of the herbivore-induced plant volatile (HIPV) blend. The backbone structure of the terpenoids is synthesized by terpene synthases (TPS). Here, we annotate the cucumber *TPS* gene family (*CsTPS* family) consisting of 27 members, much less than in for example Arabidopsis (40 TPSs) and tomato (44 TPSs). Most of the cucumber *TPSs* are located in four clusters on three of the seven chromosomes. Nineteen of these genes were isolated and heterologously expressed in *Escherichia coli*. The terpenoid products produced by the expressed proteins match the terpenoids in the spider-mite induced volatile blend as well as those emitted by roots, and male and female flowers. Analysis of the expression of *CsTPS* genes in leaves infested by spider mites demonstrated that seven of the *CsTPSs* were significantly upregulated upon herbivory. Spider-mite infestation of cucumber leaves resulted in induced terpenoid emission within 24 hours. The volatile blend emitted from male flowers changed upon spider-mite folivory, while that of female flowers was not significantly altered. Collectively, this study presents detailed insight into cucumber TPSs and their genome organisation, as well as the involvement of the *TPS* gene family in the emission of herbivore-induced plant volatiles by cucumber plants.

Keywords: Cucumber, spider mites, CsTPSs, terpenes

Introduction

Terpenoids represent a class of organic compounds consisting of 5-carbon isoprene units. Up to date more than 40,000 different structures are known in nature and these can be classified by the number of isoprene units in the molecule (Aharoni et al., 2005). However, each species is only able to synthesize a limited number of terpenes, and usually within a plant family or species specialized terpenes are formed which contribute to the plant phenotype and may allow the plant to adapt to environmental conditions, while many of them are not generally shared by all plant species (Pichersky et al., 2006, Chen et al., 2011). Although some terpenes have a function in primary plant metabolism, such as gibberellins, most of the terpenes have various roles in the interaction with their biotic and abiotic environments. They may be involved in direct plant defence against pathogens or repel herbivores (Bohlmann et al., 2000, Balkema-Boomstra et al., 2003, Wang et al., 2004, Nagegowda, 2010). Furthermore, terpenes emitted from plants may attract pollinators or enhance plant indirect defence, by mediating the attraction of parasitoids or predators of herbivores (Pichersky and Gershenzon, 2002, Degenhardt et al., 2003, Kappers et al., 2005). Terpenes have been reported to be predominantly present in the blend of leaf emitted volatiles after herbivory, wounding or treatment with elicitors or jasmonic acid (Bohlmann et al., 2000, Herde et al., 2008, Cao et al., 2010).

Terpenes with either 10, 15 or 20 carbons (C10, monoterpenes; C15, sesquiterpenes; C20, diterpenes) are synthesized by terpene synthases (TPSs) (Bohlmann et al., 1998). The genes encoding these enzymes are structurally related and constitute a medium-sized gene family that occurs across the plant kingdom, as evident from the various sequenced plant genomes (Chen et al., 2011). Although TPSs are capable to produce many thousands of different terpenes, the substrates they use are limited to geranyl diphosphate (GPP), nerolidyl diphosphate (NPP), *cis*- and *trans*- farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) (Takahashi and Koyama, 2006, Tholl, 2006). Structurally, TPSs identified from different plant species can be divided into seven subfamilies (TPS-a, b, c, d, e/f, g and h) based on their amino acid sequence (Chen et al., 2011).

Conserved motifs within the TPSs have been well studied. Many TPSs carry an RRX_8W motif at the N-terminal, which is characteristic for TPS-b and TPS-d subgroups (Bohlmann et al., 1998, Aubourg et al., 2002) and may function in the cyclization of monoterpenes (Whittington et al., 2002, Hyatt et al., 2007). Usually, monoterpene and diterpene but not sesquiterpene and triterpene synthases have plastid transit peptides upstream of this motif and these peptides are cleaved off upon import in the plastids to produce the mature proteins (Chen et al., 2011). At the C-terminal of TPSs, the highly conserved motif DDXXD encodes the active site of the metal dependent ionization of the substrates (Starks et al., 1997). Also other motifs such as DXXD, EDXXD, NSE/DTE and RXR were reported and potentially contribute to the functionality of TPSs (Cao et al., 2010, Koksal et al., 2011).

To understand the biological functions of the TPSs found in a plant species, one needs to study which terpenes can be synthesized as well as where and when they are expressed in the plant. For example, the Arabidopsis genome contains approximately 32 genes that encode functional TPSs and 14 of them have been functionally characterized, including two genes that encode diterpene synthases and 12 that encode enzymes for biosynthesis of various other terpenes (Chen et al., 2011). Expression profiles of these genes have been well studied in different tissues of Arabidopsis, including leaves and flowers (Chen et al., 2011). Transcripts of TPS genes were reported to be up-regulated by herbivory in various species such as tomato (Kant et al., 2004), maize (Schnee et al., 2002) and legumes (Arimura et al., 2004). In cucumber (*Cucumis sativus*), the expression of a gene encoding a dual (*E*)-β-OCIMENE/(*E,E*)-α-FARNESENE SYNTHASE was up-regulated by feeding by *Tetranychus urticae* spider mites (Mercke et al., 2004).

Terpenoids, including (*E*)-β-ocimene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E,E*)-TMTT] in the volatile blend emitted from cucumber were found to be essential for the attraction of *Phytoseiulus persimilis*, predators of spider mites (Dicke and Sabelis, 1988, Kappers et al., 2010, Kappers et al., 2011, Dicke et al., 1990). To date, only two TPSs have been reported in cucumber, the (*E*)-β-OCIMENE/(*E,E*)-α-FARNESENE SYNTHASE mentioned above and an (*E*)-β-CARYOPHYLLENE SYNTHASE (Mercke et al., 2004). Now the full genome of cucumber has been published (Huang et al., 2009), new possibilities arise to identify the complete cucumber TPS family. Here, we report the identification and functional characterization of the CsTPS family consisting of 27 members. We isolated and characterized 19 *CsTPS* genes which appear to encode functional enzymes in different organs, including leaves and flowers. qRT-PCR analysis showed that transcripts of six *CsTPSs* were upregulated by spider-mite herbivory suggesting they have a role in plant defence. The TPSs encoded by the genes of which transcripts were induced by spider-mite infestation produced terpenoids that were present in the volatile blend emitted by spider-mite infested cucumber leaves and the timing of induction coincided with that of volatile release. Furthermore, spider-mite folivory resulted in altered *TPS* transcripts and in changes in the terpenoids present in the volatile blend of the flowers.

Results

Genome-wide identification of the *TPS* gene family in *Cucumis sativus*

To annotate the members of the *TPS* gene family in cucumber, we used the second version of the annotated genome of domesticated cucumber [v2 assembly, http://www.icugi.org; (Huang et al., 2009)]. Thirty-four gene models were identified as putative *TPS* genes using InterProScan (Zdobnov and Apweiler, 2001) by searching for putative proteins that contained at least one of the domains: IPR001906 Terpene synthase-like, IPR005630

Terpene synthase metal-binding domain, IPR008930 Terpenoid cylases/protein prenyltransferase alpha-alpha toroid and IPR008949 Terpenoid synthase.

Table 1. List of terpene synthase genes in the cucumber genome and their location, exon number, feature motifs, size of protein and whether the gene was cloned.

				Motif					
Gene No.	Gene ID	Chromosome	Exon number	"RRX$_8$W"	"DDXXD"	"RXR"	"NSE/DTE"	Protein size (aa)	Cloned
CsTPS1	*Csa1G066550*	1	7		DDIYD	RHQ	DDLGSAKDENQEGY	598	Yes
CsTPS2	*Csa1G066560*	1	7		DDMFD	RDQ	DDLGSAKDENQEGY	555	Yes
CsTPS3	*Csa1G068570*	1	7		DDLFD	RDQ	DDLGTAKDEKQEGR	574	Yes
CsTPS4	*Csa2G298300*	2	8	RQSANYQSPLW	DDVYD	RDQ	NDLLTPLDKQNIGD	614	Yes
CsTPS5	*Csa2G298310*	2	8	RQCANYSPPLW	DDVYD	RDQ		708	Yes
CsTPS6	*Csa2G298320*	2	7				NDLASSSEEGKRGD	494	No
CsTPS7	*Csa2G299330*	2	5				NDLASSSEEAKRGE	435	No
CsTPS8	*Csa2G299350*	2	6		DDVYD	RNR	DDLGTSSDELKRGD	512	No
CsTPS9	*Csa2G299870*	2	7	RRSANYQPPIW	DDVYD	RDR	NDLASSSEEAKRGE	601	Yes
CsTPS10	*Csa2G299890*	2	7	RRSGNYQPPFW	DDVYD	RDR	DDLGTSSNELARGD	599	Yes
CsTPS11	*Csa2G299920*	2	7	RRSGNYQPSTW	DDVYD	RDR	DDLGTSSDELERGD	600	Yes
CsTPS12	*Csa2G427840*	2	7	RRIEYHEPSAW	DDMYD	RDR	NDIVSHKFGEERCH	570	Yes
CsTPS13	*Csa3G021130*	3	7	RRSANFPPNIW	DDIYD	RDR	NDIVSHKFEQERGH	553	Yes
CsTPS14	*Csa3G039850*	3	7	RRVAKYSPPPCAW	DDVYD	RDR	NDITSHKYEQKRGH	562	Yes
CsTPS15	*Csa3G040850*	3	7		DDMYD	RDR	NDITSHKFGQEREH	568	Yes
CsTPS16	*Csa3G040860*	3	6		DDMYD	RDR	NDIASHKFEQERGH	529	No
CsTPS17	*Csa3G041370*	3	7	RRTAKFPPSPW	DDTYD	RDR	NDITSHQFEQQRGH	567	Yes
CsTPS18	*Csa3G042380*	3	8	RRAAEFPPSPW	DDMYD	RVR	NDIVSHKFEQERGH	503	Yes
CsTPS19	*Csa3G095040*	3	7	RRSAQFQASVW	DDIYD	RNR	NDIVSHKFEQERGH	561	Yes
CsTPS20	*Csa3G096040*	3	5					313	No
CsTPS21	*Csa3G097040*	3	7	RSLANFHPTIW	DDIYD	RDR	DDVVSYKFEKEREH	567	Yes
CsTPS22	*Csa3G097540*	3	7	RSLANFHPTIW	DDIYD	RDR	DDIASHKFEQEREH	527	Yes
CsTPS23	*Csa6G410650*	6	14					782	Yes
CsTPS24	*Csa7G239640*	7	11				NDTRTFDRESSEGK	762	Yes
CsTPS25	*Csa1G070070*	1	1					86	No
CsTPS26	*Csa1G070080*	1	2					217	No
CsTPS27	*Csa7G025710*	7	6					170	No

The genome regions containing these genes (introns and exons) and their flanking sequences were re-annotated using FGENESH (www.softberry.com) and Genewise (www.ebi.ac.uk/) in order to get the full-length sequences of these genes. Twenty-four full length *TPS* gene models were extracted that encode putative proteins with 313 to 782 amino acids and at

least 5 exons and we renamed them as *CsTPS1-24* according to their chromosomal position (Fig. 1). Proteins encoded by *CsTPS6, CsTPS7, CsTPS8, CsTPS18* and *CsTPS20* are clearly smaller than those of other *CsTPSs*, i.e. they have less than 520 amino acids, but these genes do contain at least 5 exons (Table 1). Three gene models seem too short to encode a functional TPS protein and have less than 5 exons but because they contain some of the features of TPS domains we included them as *CsTPS25-27* (Table 1).

Twenty-two of the 27 *CsTPS* genes were found in four clusters located on chromosomes I, II and III suggesting multiple duplication and recombination events on these chromosomes (Fig. 1). *CsTPS1* and *CsTPS2* are located in tandem on chromosome I, followed by a hypothetical other gene and *CsTPS3*. After another, non-annotated gene, the partial *TPS* genes *CsTPS25* and *CsTPS26* are also located in this cluster. *CsTPS4-11* form a large TPS gene cluster on chromosome II. Only a TATA modulatory factor element and a number of other putative gene ORFs were found in this *TPS* cluster. In particular, a predicted cytochrome P450 gene (P450 78A subfamily) is located upstream of this *TPS* cluster. *CsTPS12* is located on an isolated position further downstream on chromosome II. Chromosome III harbours two *TPS* gene clusters with an interval of approximately 2 Mb in between. These two clusters contain *CsTPS14-18* and *CsTPS19-22*, respectively. *CsTPS13* is located upstream of both clusters on chromosome III. No *TPS* genes were found on chromosomes IV and V. Chromosome VI and chromosome VII both contain a single full length TPS gene (*CsTPS23* and *CsTPS24* respectively, and the partial *CsTPS27* on chromosome VII).

Phylogenetic and structure analysis

Phylogenetic analysis of the 24 potentially functional *CsTPSs* showed that five of the seven *TPS* sub-clades are present in *C. sativus* (Fig. 2). The majority of cucumber *TPSs* are classified as *TPS*-a (8 members) and *TPS*-b (11 members). Interestingly, genes *CsTPS4-11* are located in one cluster on chromosome II and *CsTPS4-11* all belong to *TPS*-a, whereas the genes in the TPS-b subclade consist of *CsTPS12*, located on chromosome II and *CsTPS13-22* that are all located on chromosome III. The *TPS*-g subclade is formed by *CsTPS1-3* that together with partial TPS genes *CsTPS25* and *CsTPS26* are located in one cluster on chromosome I. Furthermore, *CsTPS23* and *CsTPS24* are classified as TPS-c and TPSe/f, respectively, and most likely encode COPALYL DIPHOSPHATE SYNTHASE and KAURENE SYNTHASE.

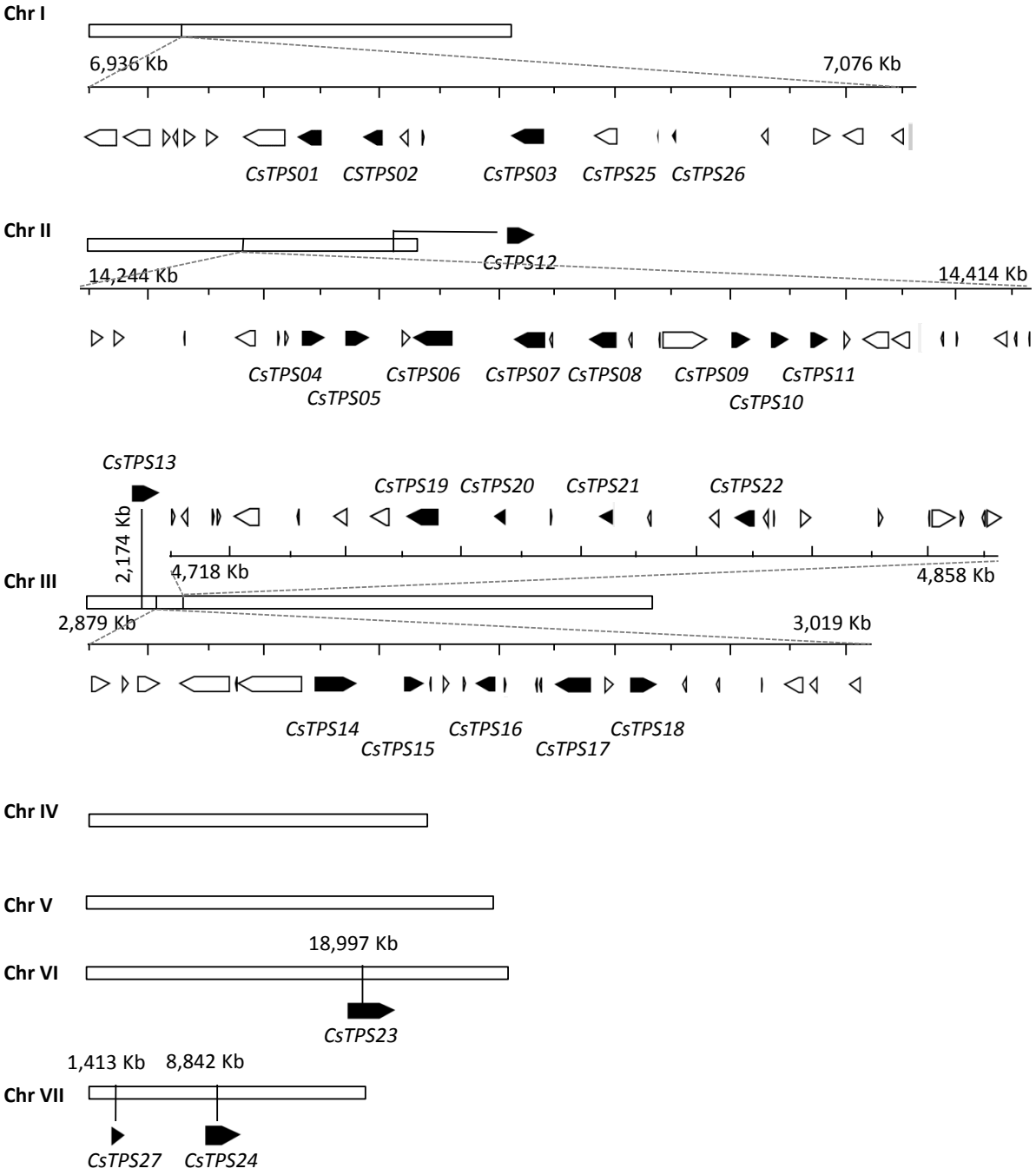

Fig. 1. Distribution of *CsTPS* genes in the cucumber genome. The location of all *CsTPS* genes is indicated at the relative position in seven artificial chromosomes (white bars); the loci harbouring *CsTPS* genes are enlarged and gene models are shown in black (*CsTPS* genes) or white (non-*CsTPS* genes), with the point representing the orientation of the genes and the Kb number indicating the position in the artificial chromosomes.

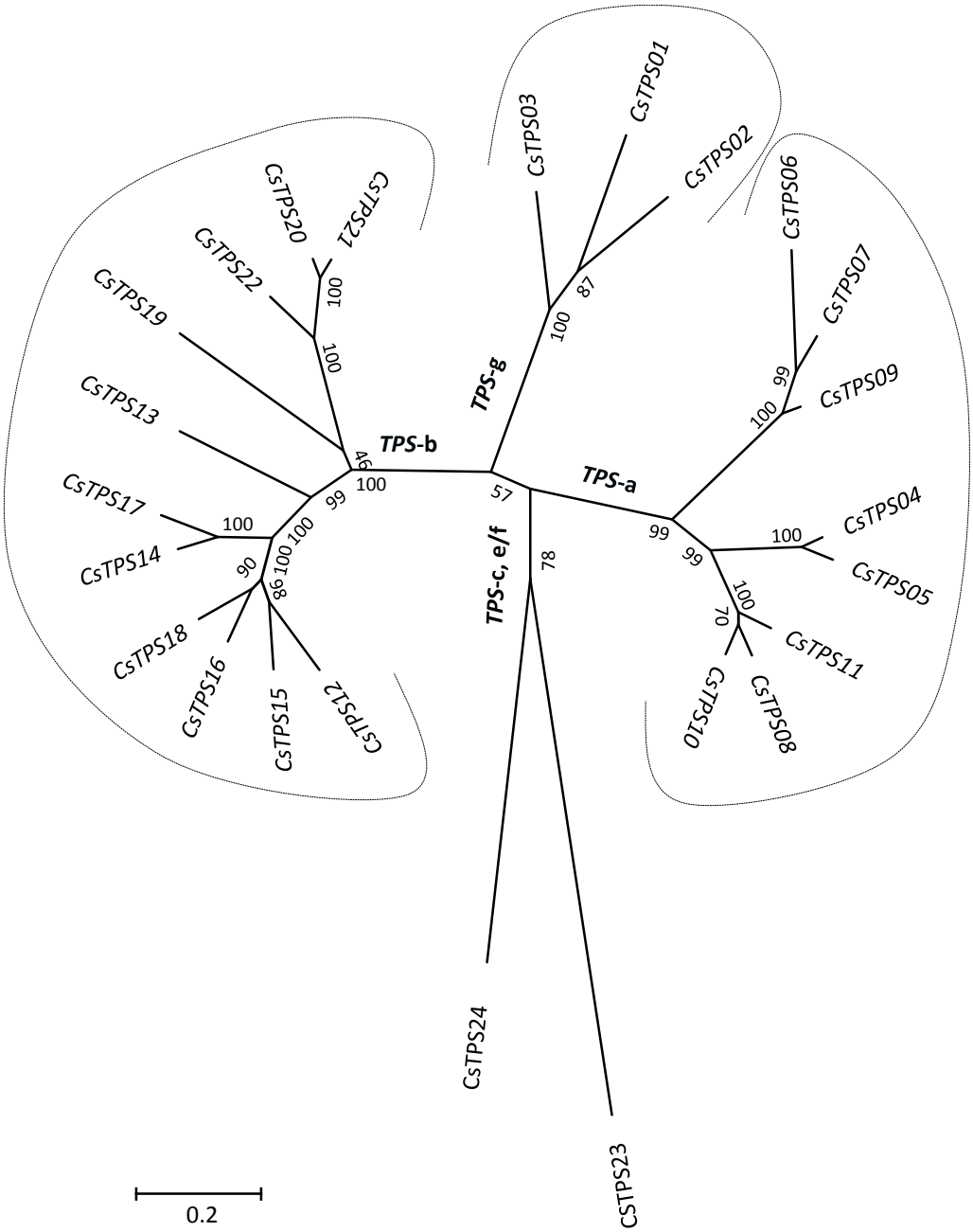

Fig. 2. Phylogenetic relationship of full length *CsTPS*s from *Cucumis sativus*. *CsTPSs* with putative full-length sequences are sub-grouped into *TPS*-a through *TPS*-f according to their sequence similarity to reported *TPS*s in Arabidopsis.

Amino acid sequence comparison

Using cDNA synthesized from RNA isolated from jasmonic acid-treated cucumber (variety Chinese long 9930) leaves, roots or flowers, we amplified and cloned 19 full length open reading frames of *CsTPS* genes. Two of them (renamed as *CsTPS19* and *CsTPS21*) were also isolated and characterized before by Mercke et al. (2004) and encode (*E*)-β-OCIMENE/(*E,E*)-*α*-FARNESENE SYNTHASE and (*E*)-β-CARYOPHYLLENE SYNTHASE, respectively.

The full-length proteins of the whole CsTPS family were aligned using ClustalW (Thompson et al., 1994). Based on sequence similarity to representative TPSs from Arabidopsis, CsTPS1-11 were tentatively classified as monoterpene synthases, CsTPS12-22 as sesquiterpene synthases and CsTPS23 and CsTPS24 as diterpene synthases. CsTPS1-24 all have a length between 313 and 813 amino acids and contain typical elements known to be conserved in terpene synthases including the DDXXD motif and the NSE/DTE motif (Table 1). The RRX_8W motif, a typical sequence motif in the N-terminal part is present in CsTPS9-13, 17-19, but is altered to RQX_8W in CsTPS4 and CsTPS5, to RSX_8W in CsTPS21 and CsTPS22, to $RRX_{10}W$ in CsTPS14 and is completely absent in CsTPS1-3, CsTPS6-8, CsTPS16 and CsTPS20. The RXR motif is present in most potential full-length CsTPSs but absent in CsTPS6-7, CsTPS20, CsTPS23 and CsTPS24. Most of the proteins contain motif DDXXD, except CsTPS6 and CsTPS7. In CsTPS23 a DXDD motif was present and in CsTPS24 a DDXXD, but the complete sequence did not align well with other CsTPSs and hence they form a separate group. The putative proteins encoded by the partial genes CsTPS25, CsTPS26 and CsTPS27 contain none of the motifs mentioned above; however, they did align to the TPS feature protein domains IPR001906, terpene synthase-like, or IPR005630, terpene synthase metal-binding domain.

Using the TargetP1.1 server (Emanuelsson et al., 1999), the N-terminal sequence upstream of the RRX_8W motif was analysed for the presence of signal peptides which could target the proteins to specific subcellular locations. A plastid transit peptide was found for CsTPS1-3, CsTPS9-11, CsTPS15 and CsTPS24 - supporting their putative role as monoterpene synthases and diterpene synthase in the case of CsTPS24 - and a mitochondrial targeting peptide was found for CsTPS4. A secretory pathway signal peptide was found for CsTPS5. In contrast, no signal peptides were found for the putative monoterpene synthases CsTPS6-8, CsTPS12-14, CsTPS16-22 and diterpene synthase CsTPS23.

Product characterization of cucumber TPSs *in vitro*

Nineteen terpene synthase genes were successfully isolated and subsequently cloned into the pACYCDuet expression vector for heterologous expression of the proteins (Table 2). All tested proteins except CsTPS13 and CsTPS18 accepted substrates GPP and FPP resulting in the formation of various mono- and sesquiterpenes, respectively. CsTPS proteins with a

predicted chloroplast target peptide efficiently produced one or multiple monoterpenoids as the ratio of the sum of the peak areas of all monoterpene products to that of all monoterpenes including the non-specific geraniol was more than 50% (Fig. 3). These enzymes also catalysed the formation of sesquiterpenes from FPP, but the sum of the peak areas of all sesquiterpene products together was less than the amount of non-specific farnesols.

CsTPS1-3 have a predicted chloroplast target peptide and produced predominantly linalool when GPP was supplied as substrate (Fig. 3). Other products formed by these enzymes were β-myrcene, limonene and α-terpineol in the case of CsTPS1, and a trace of limonene for CsTPS3. CsTPS2 only produced linalool, however, it was not able to convert all the GPP as the ratio linalool to non-specific geraniol was only around 50% (Fig. 3). Four other CsTPSs have predicted chloroplast target peptides. CsTPS9 catalysed the formation of (*E*)-β-ocimene and β-myrcene in a ratio of 75:25 from GPP and only traces of non-specific geraniol were found in these assays, indicating an efficient conversion under these assay conditions (Fig. 3). CsTPS11 and CsTPS15 both catalysed the formation of β-myrcene, limonene, (*E*)-β-ocimene, linalool and α-terpineol from GPP but to different amounts and CsTPS11 additionally catalysed the formation of an unidentified monoterpene from GPP (Fig. 3). The product profile of CsTPS10 was distinct from the other chloroplast-targeted enzymes because it produced α-pinene, α-phellandrene, sabinene, β-pinene, α-terpineol, β-myrcene and linalool from GPP (Fig. 3, Fig. 4).

Table 2. Putative localization and the major products of enzymatic activity in vitro of CsTPS.

Acronym	Cloned	Putative localisation	Major product	Name	Substrates tested*					Category
					GPP	NPP	(*E*,*E*)-FPP	(*Z*,*Z*)-FPP	GGPP	
CsTPS01	Yes	Chloroplast	(S)-linalool	CsLIN1	A	A	A	A	N	mTPS
CsTPS02	Yes	Chloroplast	(S)-linalool	CsLIN2	A	A	A	N	N	mTPS
CsTPS03	Yes	Chloroplast	(S)-linalool	CsLIN3	A	A	A	A	N	mTPS
CsTPS04	Yes	Mitochondria	(*E*)-β-farnesene	CsβFAR1	A	A	A	N	N	sTPS
CsTPS05	Yes	Secretory pathway	(*E*)-β-ocimene	CsβOCI1	A		A		N	mTPS
CsTPS06	No	Cytosol	not analysed							
CsTPS07	No	Cytosol	not analysed							
CsTPS08	No	Cytosol	not analysed							
CsTPS09	Yes	Chloroplast	(*E*)-β-ocimene	CsβOCI2	A	N	A	N	A	mTPS
CsTPS10	Yes	Chloroplast	multiproduct monoterpene	CsmTP1	A		A		N	mTPS
CsTPS11	Yes	Chloroplast	(S)-linalool / β-myrcene	CsLIN/MYR1	A		A		N	mTPS
CsTPS12	Yes	Cytosol	(*E*)-β-farnesene	CsβFAR2	A		A		N	sTPS
CsTPS13	Yes	Cytosol	not active		N		N		N	
CsTPS14	Yes	Cytosol	(*E*)-β-farnesene	CsβFAR3	A	A	A	A	N	sTPS
CsTPS15	Yes	Chloroplast	(S)-linalool / (*E*)-β-ocimene	CsLIN/βOCI1	A	A	A	A	N	mTPS
CsTPS16	No	Cytosol	not analysed							
CsTPS17	Yes	Cytosol	(*E*)-β-farnesene / (*E*)-nerolidol	CsFAR/NER1	A		A		N	sTPS
CsTPS18	Yes	Cytosol	not active		N		N		N	
CsTPS19	Yes	Cytosol	(*E,E*)-α-farnesene	CsαFAR1	A	A	A	A	N	sTPS
CsTPS20	No	Cytosol	not analysed							
CsTPS21	Yes	Cytosol	(*E*)-β-caryophyllene	CsβCAR1	A	A	A	A	N	sTPS
CsTPS22	Yes	Cytosol	τ-cadinol	CsτCAD1	A	A	A	A	N	sTPS
CsTPS23	Yes	Cytosol	??		A		A		N	sTPS
CsTPS24	Yes	Chloroplast	geranyl linalool	CsGL1	A		A		A	dTPS

*: A: accepted; N: not accepted; Blank: not tested. mTPS: monoterpene synthase; sTPS: sesquiterpene synthase; dTPS: diterpene synthase.

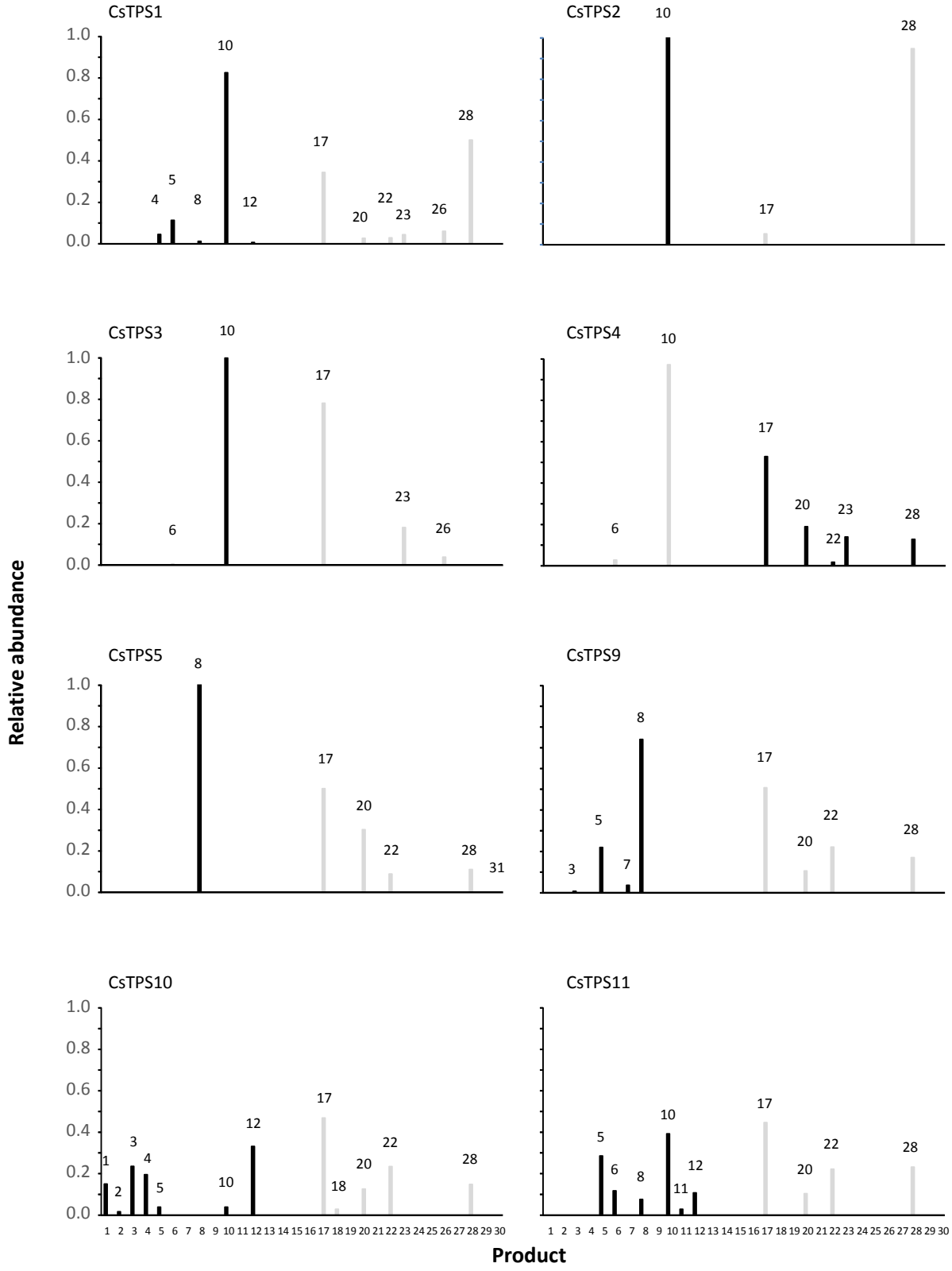
CsTPS1
CsTPS2
CsTPS3
CsTPS4
CsTPS5
CsTPS9
CsTPS10
CsTPS11
Relative abundance
Product

Fig. 3 (part 1)

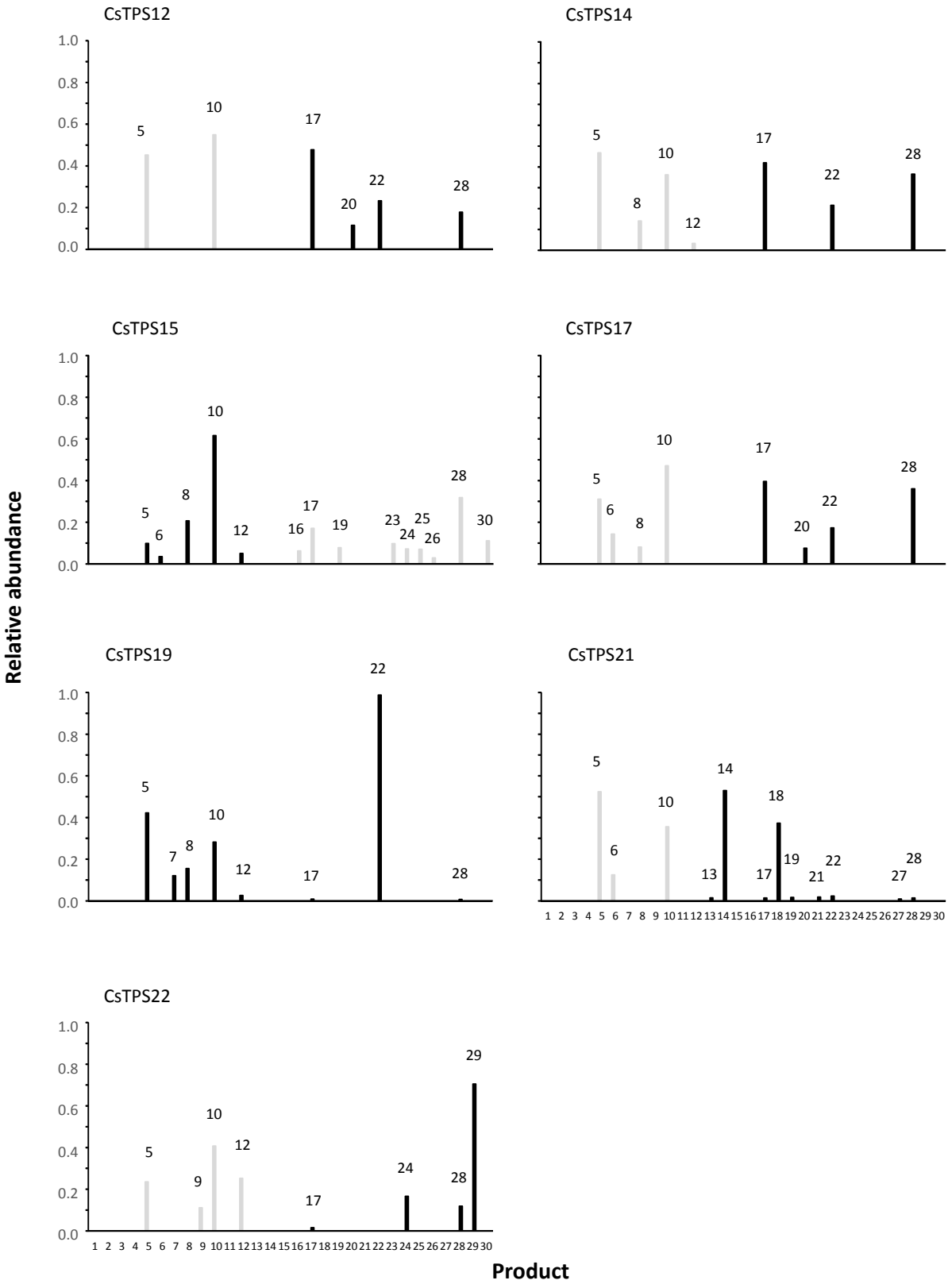

Fig. 3 (continued): Products formed *in vitro* by CsTPSs. Enzymes were incubated with GPP or FPP and products were analysed as described in Materials and Methods. Reaction products (peaks) were identified by comparison of their GC-MS mass spectra with NIST (version 2011) and in-house libraries and their retention indices with those in Adams et al (2007). Compounds are indicated in numbers above the bars and on the X-axis: 1 α-pinene, 2 α-phellandrene, 3 sabinene, 4 β-pinene, 5 β-myrcene, 6 limonene, 7 (*Z*)-β-ocimene, 8 (*E*)-β-ocimene, 9 terpinolene, 10 linalool, 11 unknown monoterpene (calculated retention index: 1169.3), 12 α-

terpineol, 13 β-elemene, 14 (*E*)-β-caryophyllene, 15 (*Z*)-β-farnesene, 16 α-bergamotene, 17 (*E*)-β-farnesene, 18 α-humulene, 19 unknown sesquiterpene (calculated retention index: 1485.1), 20 (*Z,E*)-α-farnesene, 21 unknown sesquiterpene (calculated retention index: 1500.1), 22 (*E,E*)-α-farnesene, 23 β-bisabolene, 24 δ-cadinene or τ-cadinene, 25 (*Z*)-nerolidiol, 26 (*Z*)-α-bisabolene, 27 unknown sesquiterpene (calculated retention index 1556.3), 28 (*E*)-nerolidol, 29 τ-cadinol, 30 *epi*-α-bisabolol, 31 geranyl linalool. Depicted are the relative abundances of all monoterpenes in case of incubation with GPP (i.e. sum of all monoterpenes is 1) and all sesquiterpenes in case of incubation with FPP. Peak areas were calculated using the TIC, Total Ion Chromatogram. Black bars indicate whether preferably monoterpenes or sesquiterpenes are produced by each of the enzymes, based on the ratio of all products formed to that of all products plus aspecific products and grey bars indicate that the enzyme also uses the other substrate, but with lower efficiency as the sum of the peak areas for all products relative to the sum of all products including aspecific products is < 50%. For example in the case of CsTPS1, the black bars indicates that GPP was used to produce linalool, β-pinene, β-myrcene and traces of (*E*)-β-ocimene and α-terpineol, while the grey bars indicate that FPP was also accepted as substrate by CsTPS1, but that the total peak area of sesquiterpene products was less than the peak area of aspecifically produced farnesols.

Despite the presence of a predicted chloroplast target peptide, CsTPS1, CsTPS2 CsTPS3, CsTPS9, CsTPS10 and CsTPS11 catalysed the formation of various sesquiterpenes from FPP (Fig. 3, Fig. 5), including (*E*)-β-farnesene, (*Z,E*)-α-farnesene, (*E,E*)-α-farnesene, (*E*)-nerolidol and β-bisabolene. CsTPS15 produced quite a different product profile, with α-bergamotene, (*E*)-β-farnesene, β-bisabolene, δ-cadinene, (*Z*)-nerolidol, (*Z*)-α-bisabolol, (*E*)-nerolidol and an unknown sesquiterpene (retention index 1485). However, in all these assays the conversion of FPP was less efficient than the conversion of GPP as the sum of the peak areas for the sesquiterpenes was less than 40% of the total peak area including the non-specific conversion product farnesol. In addition, CsTPS9 accepted GGPP as a substrate resulting in the formation of a small amount of geranyl linalool (Fig. 3).

We were not able to successfully clone *CsTPS6*, *CsTPS7*, *CsTPS8*, *CsTPS16* and *CsTPS20*, all genes without a targeting sequence. CsTPS13 and CsTPS18 were not active in any of the assays that we performed (Table 2). Other proteins without a predicted targeting peptide were CsTPS12-14 and CsTPS17 that all catalysed the formation of (*E*)-β-farnesene, and to a lesser extent (*E,E*)-α-farnesene and (*E*)-nerolidol from FPP. Furthermore, minor amounts of (*Z,E*)-α-farnesene were formed by CsTPS12 and CsTPS15. CsTPS19 predominantly catalysed the formation of (*E,E*)-α-farnesene and CsTPS21 catalysed the formation of (*E*)-β-caryophyllene and α-humulene from FPP, while the major product of CsTPS22 was τ-cadinol. When cytosolic terpene synthases were supplemented with GPP, most of the enzymes produced small amounts of β-myrcene and linalool, however the total peak area of these products was in all cases less than 20% of that of the non-specific conversion product geraniol. An exception was CsTPS19 that accepted GPP efficiently to catalyse the formation of (*E*)-β-ocimene, showing that CsTPS19 is an efficient dual (*E*)-β-OCIMENE/(*E,E*)-α-FARNESENE SYNTHASE (Fig. 3, Fig. 5).

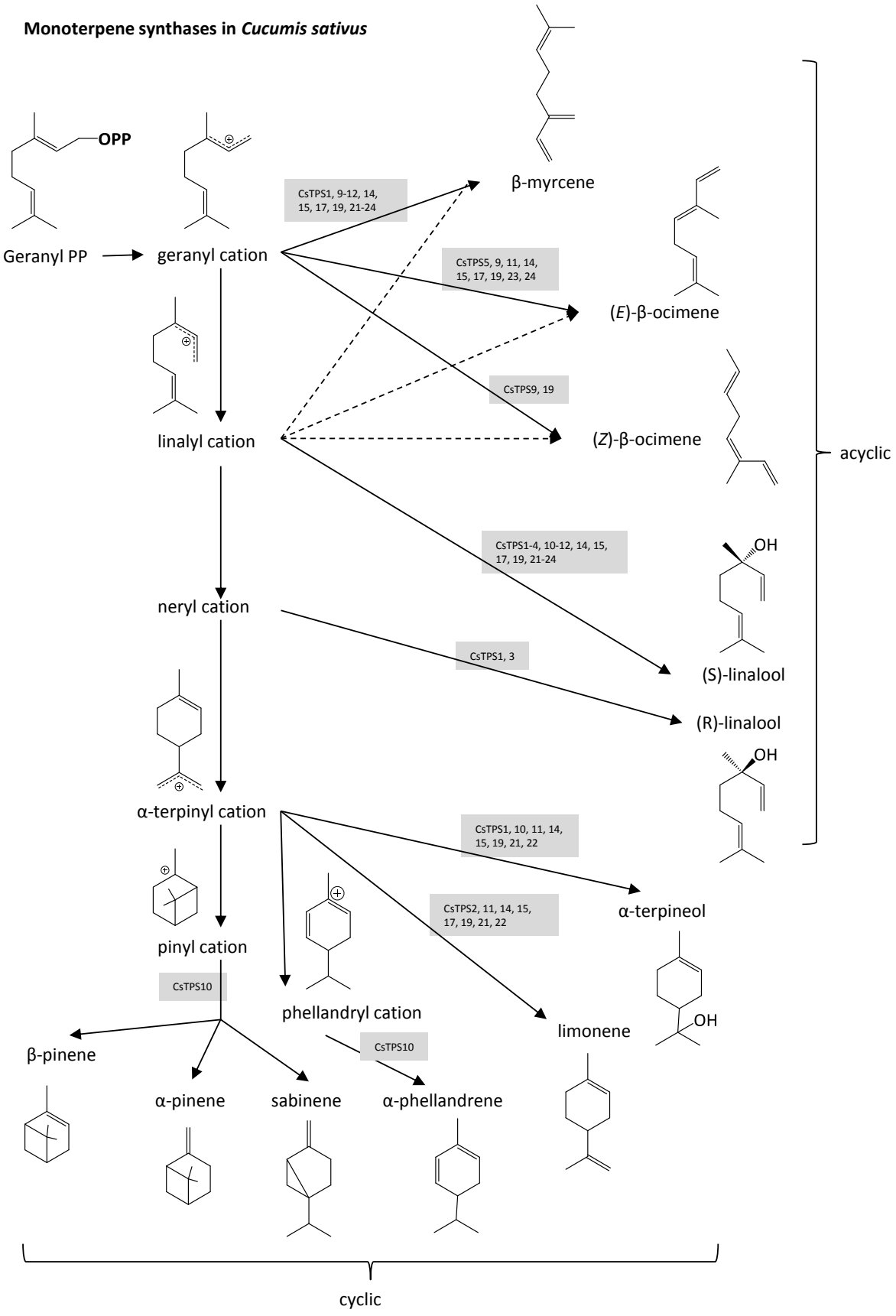

Fig. 4. Overview of monoterpenes formed *in vitro* by cucumber terpene synthases (TPSs). Arrows indicate probable conversion steps and CsTPSs catalysing these steps are depicted in grey boxes.

Sesquiterpene synthases in *Cucumis sativus*

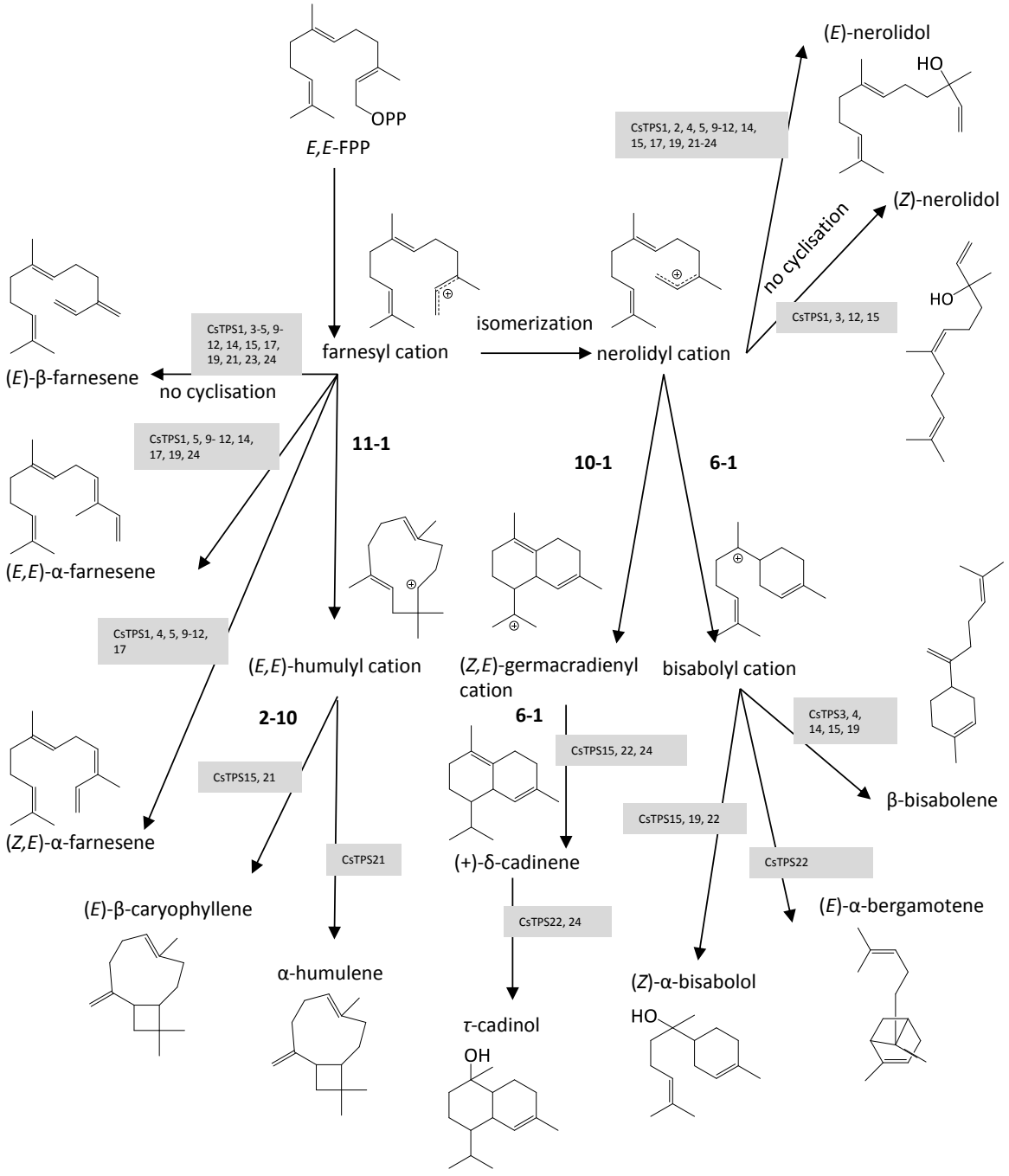

Fig. 5. Overview of sesquiterpenes formed *in vitro* by cucumber terpene synthases (TPSs) from (*E,E)*-farnesyl diphosphate (FPP). Arrows indicate probable conversion steps and CsTPSs catalysingt thyese steps are depicted in grey boxes.

Two CsTPS have a distinct predicted targeting; CsTPS4 contains a predicted mitochondrial target peptide and CsTPS5 is predicted to be targeted to the secretory pathway. These enzymes are designated as (*E*)-β-FARNESENE SYNTHASE and (*E*)-β-OCIMENE SYNTHASE respectively, based on the most intense products formed (Fig. 3).

Table 3. The abundance of *CsTPS* genes transcripts (RPKM) in cucumber leaves of Chines Long (Cl) and Corona (Co) non-infested or infested for one, two and three days with spider mites. Transcript abundance was analysed by RNA-seq. Colour coding: white represents low expression and red represents high expression relative to the average expression of the gene.

Gene No.	Cl day 0	Cl day 1	Cl day 2	Cl day 3	Co day 0	Co day 2	Co day 3
CsTPS1	0.2	0.1	0.9	0.2	2.7	2.8	2.3
CsTPS2	0.4	1.6	4.3	2.2	21.7	29.3	27.2
CsTPS3	0.5	1.1	1.0	0.8	2.4	4.3	5.2
CsTPS4						0.2	0.4
CsTPS5	17.4	17.1	19.0	21.9	24.4	26.3	32.3
CsTPS6						1.1	0.7
CsTPS7						0.5	0.1
CsTPS8							
CsTPS9		1.4	2.6	3.1	0.4	5.0	6.6
CsTPS10							
CsTPS11				0.1			
CsTPS12	0.1	0.1	0.2		0.2	0.1	
CsTPS13		0.1					
CsTPS14			0.1			0.2	
CsTPS15							
CsTPS16	0.3						
CsTPS17	0.6		0.3				
CsTPS18							
CsTPS19		0.4	4.7	3.6	0.8	4.0	8.3
CsTPS20							
CsTPS21	0.2	0.1	0.2	0.1	0.2	0.5	0.5
CsTPS22	0.1				0.1	0.2	
CsTPS23	1.3	1.0	1.4	0.9	1.0	1.3	0.6
CsTPS24	0.2	0.2	0.1		0.2	0.1	

Although a number of CsTPS proteins could convert *cis*-substrates NPP and (*Z,Z*)-FPP into various terpenoids, none of the CsTPSs accepted the *cis*-substrates more efficiently than the *trans*-substrates suggesting that these substrates do not play a role in cucumber terpene biosynthesis (data not shown). Both *CsTPS23* and *CsTPS24* are predicted to encode a diterpene synthase. Both enzymes accepted GPP as a substrate to produce (*E*)-β-ocimene, linalool and β-myrcene in minor amounts. FPP was not accepted as substrate, although CsTP24 produced a small amount of *τ*-cadinol and both enzymes produced some (*E*)-nerolidol. Finally, CsTPS24, but not CsTPS23, accepted GGPP to produce geranyl linalool.

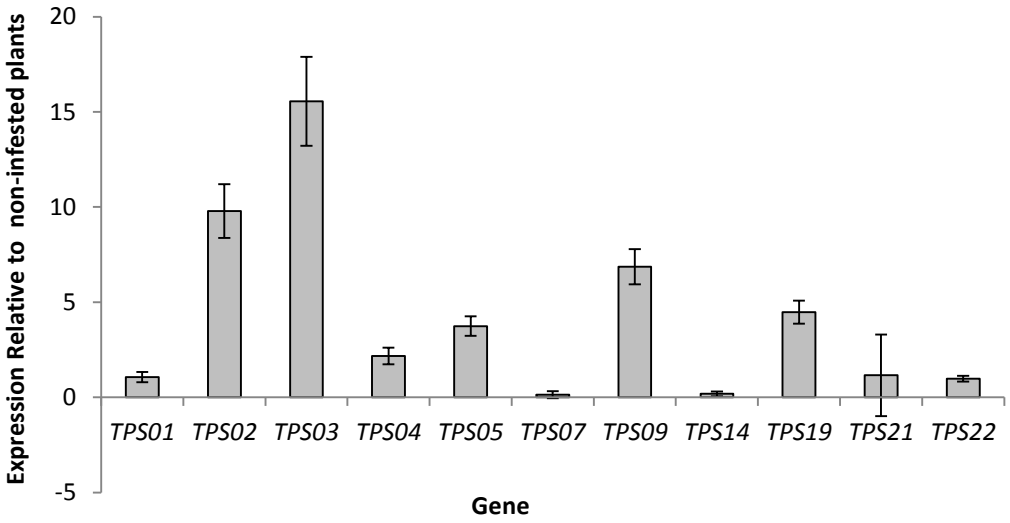

Fig. 6. Up-regulation of some *CsTPS* genes in leaves of cucumber Corona after nine days of infestation with spider mites. The cucumber *Actin* gene (*Csa6M484600*) was used as an internal control and gene expression was calculated as $2^{-\Delta\Delta Ct}$. Data represent fold expression difference of each gene in infested compared with non-infested. Error bars represent SD of three biological replicates.

Spider mites induced *CsTPS* expression and terpene volatile production

The responsiveness of *CsTPS* genes to herbivory was analysed by RNA-seq analysis of cucumber leaves that were infested by spider mites for one, two and three days (Chapter 2, this thesis). Transcripts of 19 *CsTPS* genes were detected. In non-infested leaves, transcripts of 13 *CsTPS* genes were present in cucumber accessions Chinese Long 9930 and Corona together (Table 3). Transcripts of *CsTPS16* and *CsTPS17* were only detected in Chinese Long [low RPKM (Reads Per Kilobase of transcript per Million mapped reads values)] and low levels of *CsTPS9* and *CsTPS19* transcripts were present in non-infested leaves of Corona only (Table 3). Among the detected genes in non-infested leaves, *CsTPS5* had the highest expression with 17.4 RPKM in Chinese Long and 24.4 RPKM in Corona. The transcript level of *CsTPS2* was relatively high (21.7 RPKM) in accession Corona, while only 0.4 RPKM in accession Chinese long. Expression levels of all other *CsTPS* genes were low, with transcripts less than 1.3 RPKM (2.54% of the average overall expression) in Chinese Long and less than 2.7 RPKM (5.81% of the average overall expression) in Corona (Table 3).

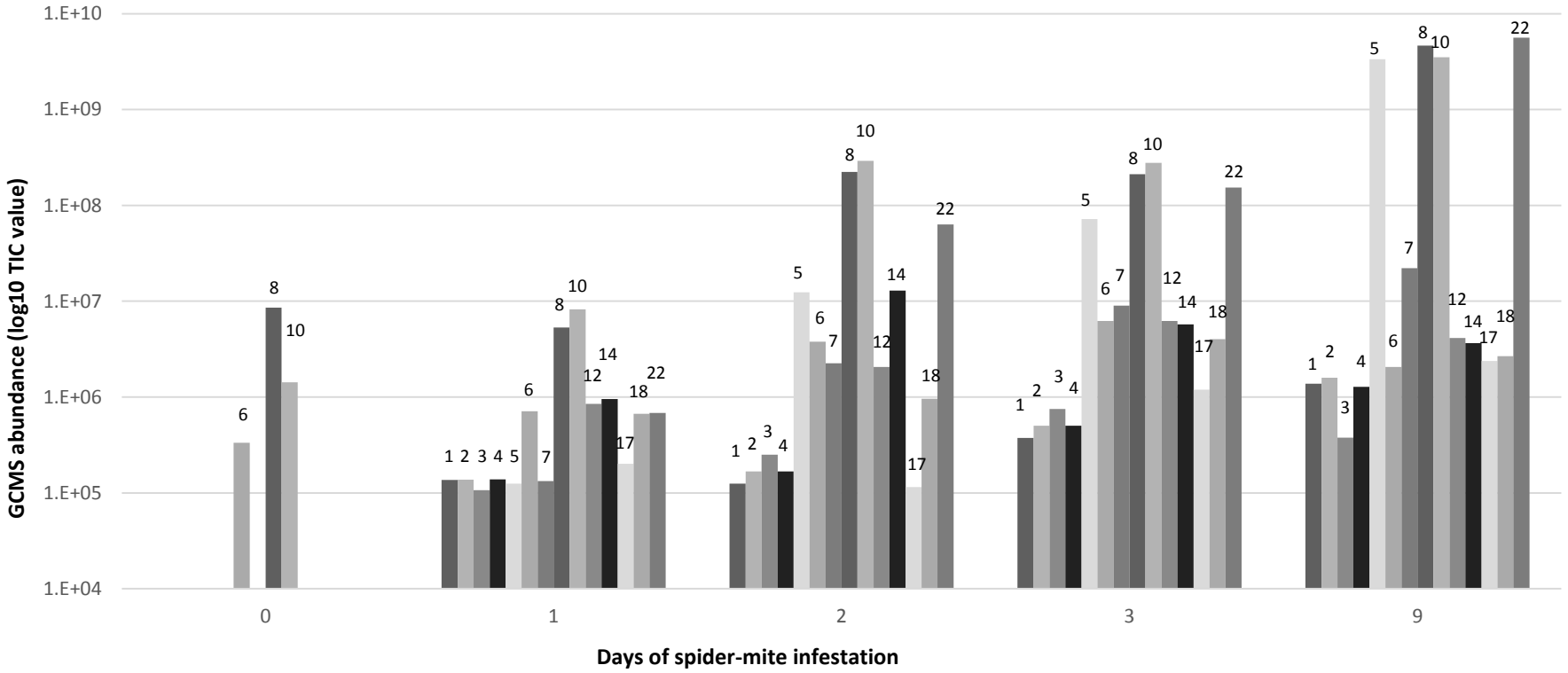

Fig. 7. Terpenoids in the blend of volatiles emitted from leaves of cucumber infested with spider mites. Compounds are indicated as numbers above the bars: 1 α-pinene, 2 α-phellandrene, 3 sabinene, 4 β-pinene, 5 β-myrcene, 6 limonene, 7 (*Z*)-β-ocimene, 8 *E*-β-ocimene, 10 linalool, 12 α-terpineol, 14 (*E*)-β-caryophyllene, 17 (*E*)-β-farnesene, 18 α-humulene, 22 (*E,E*)-α-farnesene. Compounds were identified by comparison of their mass spectra with authentic standards, NIST libraries and in-house libraries and their retention indices with those in Adams et al. (2007).

Spider-mite infestation resulted in an increase in the expression of several *TPS*s from one day of infestation onwards (Table 3). During the first three days of infestation transcripts of *CsTPS1-3* increased 2- to 10-fold compared to non-infested leaves, in both Chinese Long and Corona, and *CsTPS4* expression increased from non-detectable to 0.4 RPKM in Corona. In addition, expression of *CsTPS9* and *CsTPS19* was upregulated by spider-mite feeding (Table 3). Transcripts of both genes were not detectable in unchallenged leaves of Chinese Long but increased to 3.1 RPKM and 4.7 RPKM, respectively, upon spider-mite infestation, while in Corona transcripts increased 17- and 10-fold as a result of spider-mite infestation. Progressing infestation during three days resulted in five *TPS* genes that were predominantly expressed, i.e. the upregulated *CsTPS2, CsTPS3, CsTPS9* and *CsTPS19* and the constitutively expressed *CsTPS*5 that showed a small increase in expression in both accessions during infestation (1.3-fold).

We analysed expression of several selected *CsTPS* genes of which transcripts increased or remained stable upon progressing infestation with spider mites using qRT-PCR (Fig. 6). Transcript abundance of *CsTPS4* and *CsTPS5* increased 2- to 5-fold after nine days of mite infestation compared to non-infested leaves of the same age, while transcript abundances of *CsTPS2*, *CsTPS3, CsTPS9* and *CsTPS19* increased more than 5-fold (Fig. 6). The strongest induction was found for transcripts of *CsTPS3* that increased 15-fold after nine days of spider-mite infestation.

Changes in the presence and abundance of terpenoids were found in the volatile blend emitted from cucumber leaves that were infested by spider mites for one, two, three or nine days (Fig. 7). In the blend of volatiles emitted from non-infested cucumber leaves, only small amounts of (*E*)-β-ocimene, linalool and (*Z*)-β-ocimene were present. One day after the onset of infestation, 14 terpenes were detected in the induced volatile blend. The total amount of terpenoids increased about 2-fold on day one and 1660-fold after nine days of infestation. One day after infestation the blend mainly consisted of linalool (45% of total terpenoids) and (*E*)-β-ocimene (29%), while (*E*)-β-caryophyllene (5%), α-terpineol (5%), limonene (4%), α-humulene (4%), (*E,E*)-α-farnesene (4%), (*E*)-β-farnesene (1%), α-pinene, α-phellandrene, sabinene, β-pinene and β-myrcene (all less than 1%) were present in minor amounts. With progressing infestation, no new terpenes were detected in the headspace of spider-mite infested cucumber leaves but the contribution of individual terpenoids to the blend changed such that (*E,E*)-α-farnesene became the predominant terpenoid (33% of all terpenoids) followed by (*E*)-β-ocimene (27%), linalool (20%) and β-myrcene (20%), while all other terpene compounds contributed less than 1% of the total after nine days of infestation.

Expression of *CsTPS* genes and volatile emission in different organs of cucumber plants and responsiveness to infestation by spider mites

Exploring the RNA-seq data of different organs of unchallenged cucumber plants using published data (Li et al., 2011) revealed that expression of *CsTPS* genes varied among different organs (Table 4). The monoterpene synthase genes *CsTPS2, CsTPS11* and the sesquiterpene synthase genes *CsTPS12-14* showed complete or almost complete root-specific expression in unchallenged plants, whereas transcripts of *CsTPS15* were exclusively present in reproductive organs and in tendrils. Transcripts of 12 genes were detected in roots, 13 in stems, 14 in leaves, 13 in male - and 16 in female flowers and 15 in ovaries (Table 3). Male and female flowers are distinctive with respect to *TPS* expression. Expression levels in unchallenged plants of *CsTPS1*, *CsTPS*2, *CsTPS15*, *CsTPS19*, *CsTPS21*, *CsTPS22* and *CsTPS24* were 2-fold higher in female flowers while *CsTPS4*, *CsTPS16* and *CsTPS18* had higher expression in male flowers. Interestingly, transcripts of monoterpene synthase genes *CsTPS5* and *CsTPS9*, sesquiterpene synthase genes *CsTPS14* and *CsTPS20-22* and diterpene synthase gene *CsTPS24* were at least 2-fold higher in unfertilized ovaries (seven days after flowering) than in fertilized ovaries (seven days after flowering), while in contrast, expression of monoterpene synthase *CsTPS4* and diterpene synthase *CsTPS23* were over 2-fold higher in fertilized than unfertilized ovaries. No transcripts were detected for *CsTPS6*, *CsTPS8* and *CsTPS17* in any of the organs of (unchallenged) plants. Most *CsTPS* genes had transcript levels below 10 RPKM, which is less than 50% of the average expression of all genes (ranging from 22 to 28 RPKM in different organs). However, expression of some *CsTPS* genes was 10 RPKM or higher in roots (*CsTPS02*, *CsTPS11-14*), and female flowers and ovaries (*CsTPS15* and *CsTPS21*). Impressively, transcripts of *CsTPS11* and *CsTPS14* were 384.2 and 235.9 RPKM in (unchallenged) roots, respectively.

We analysed the volatile emission of various organs of cucumber plants that were grown in the greenhouse and not deliberately challenged by any biotic or abiotic stress. However, we did observe the presence of sciarid flies, of which the larvae are known to feed on plant roots. The non-terpenoid compounds 6-methyl 5-heptene-2-one, (*E*)-geranyl acetone and decanal were present in the headspace of all vegetative parts, including roots. In addition, flowers emitted benzyl alcohol, benzaldehyde and to a lesser extent methyl salicylate and methoxy-phenyl oxime (data not shown). With respect to terpenoid emission, linalool was the dominant compound emitted by all vegetative tissues, although absolute levels were low. The headspace of unchallenged leaves and stems contained traces of (*E*)-β-ocimene.

The headspace of male and female flowers differed both quantitatively and qualitatively (Fig. 8A). Male flowers predominantly emitted benzyl alcohol and benzaldehyde (more than 90% of the total amount of volatiles, data not shown) and only minute amounts of β-myrcene, δ-cadinene, (*E*)-nerolidol and τ-cadinol. Although the major constituents of female flowers also were benzyl alcohol and benzaldehyde (70-85% of total volatile blend), these flowers displayed a larger contribution of terpenoids within the blend (Fig. 8A). Linalool, δ-cadinene

and (*E*)-nerolidol were the most abundant terpenoids, while β-myrcene and (*E*)-β-caryophyllene were emitted in much lower amounts. Minute amounts of α-pinene, β-pinene, limonene, (*E*)-β-ocimene, terpinolene and α-phellandrene were present in the headspace of female flowers but not in male flowers. Terpenoid emission by flowers changed upon spider-mite feeding on the leaves (folivory). The headspace of male flowers of plants of which the leaves were infested with spider mites contained relatively more α-pinene, β-pinene, (*E*)-β-ocimene, linalool, α-terpineol, (*E*)-β-caryophyllene, δ-cadinene and (*E*)-nerolidol and less β-myrcene and τ-cadinol (Fig. 8). In contrast, (*E*)-β-caryophyllene and (*E,E*)-α-farnesene were slightly more abundant in female flowers on plants with spider-mite folivory while linalool emission decreased.

Non-induced roots grown in aeroponics emitted few volatiles of which α-phellandrene was the most important (Fig. 8B). Jasmonic-acid treatment of roots resulted in increased emission of terpenoids including α-pinene, α-phellandrene and (*E,E*)-α-farnesene, which were absent in untreated roots.

Table 4. Expression of *CsTPS* in different organs (RPKM values). Colour scale indicates relative abundance from white (absent) to red (high expression). Purple indicates expression levels out of range.

Gene No.	Root	Stem	Leaf	Male flower	Female flower	Ovary unfert*	Ovary fert *	Ovary	Tendril	Base tendril
CsTPS1			0.2		0.3					
CsTPS2	13.7	0.2	0.5	0.2	1.1				4.4	3.7
CsTPS3		0.2	1.6	1.4	1.6				1.8	0.8
CsTPS4	0.9		0.3	0.4	0.2	0.1	1	4		
CsTPS5	13.6	15.5	2.3	25.7	24.3	34.5	17.6	26	13.9	14.5
CsTPS6										
CsTPS7	2.2	1.8	0.1			0.1			0.1	
CsTPS8										
CsTPS9	0.3	2.7	0.4		0.2	0.2		0.1	0.2	
CsTPS10	0.5				0.1			0.3		
CsTPS11	23.4									
CsTPS12	384.2									
CsTPS13	16.3									
CsTPS14	235.9	0.1	0.1	0.1	0.2	0.5	0.1	0.1		0.1
CsTPS15				0.1	9.7	62.6	64.3	32.7	4.8	3.3
CsTPS16		0.1		0.4	0.1		0.1	0.2		
CsTPS17										
CsTPS18	0.6	1.8		2				0.2		
CsTPS19			0.6	0.3	1.8	0.4	0.4	0.3	0.1	0.2
CsTPS20		0.1	0.2		0.7	0.7	0.2	0.5	0.1	0.2
CsTPS21		1.4	2.7	2.3	36.8	36	11.9	16.2	1.5	1.3
CsTPS22		4.4	33.3	5.4	27.1	0.7	0.3	1.7	11.3	9.4
CsTPS23	0.3	1	0.2	0.5	0.5	1.1	2.8	2.5	0.3	0.4
CsTPS24	1.2	4.3	0.5	0.1	0.8	4.4	0.6	0.4	3.7	7.2

*: Ovary unfert: Ovary unfertilized (seven days after flowering); Ovary fert: ovary fertilized seven days after flowering; Ovary: ovary of flower.

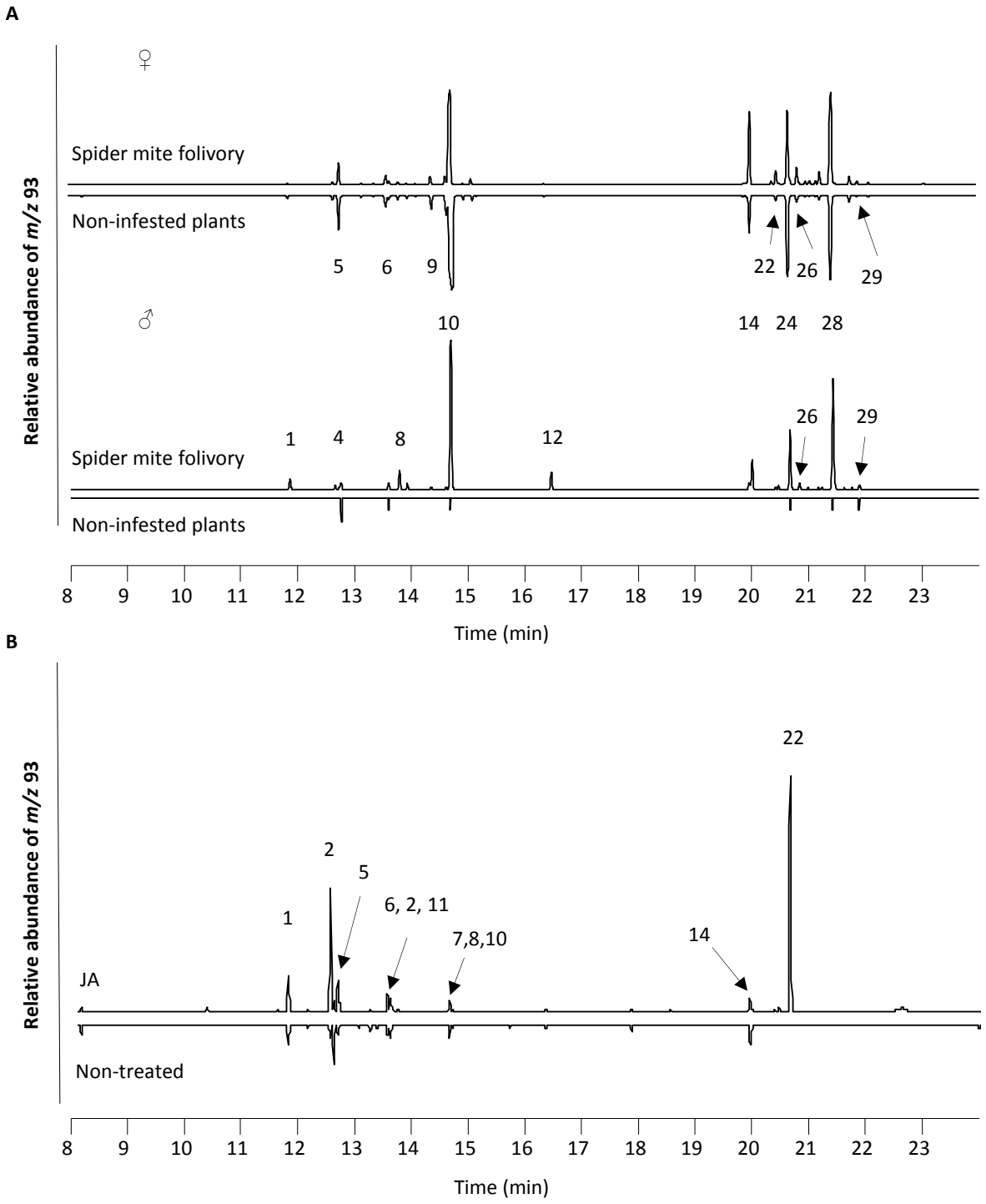

Fig. 8. GC-MS analysis of the terpenoid compounds in flowers, as affected by folivory of spider mites, and in roots as induced by JA. (**A**) Headspace of single male or female flowers, detached from plants that were either non-infested or infested with spider mites for 15 days on their leaves (folivory). (**B**) Headspace of Chinese Long roots non-treated or treated with JA for three days. Compounds were identified by comparison of their mass spectra with authentic standards and NIST libraries. 1 α-pinene, 2 α-phellandrene, 4 β-pinene, 5 β-myrcene, 6 limonene, 8 *E*-β-ocimene, 9 terpinolene, 10 linalool, 12 α-terpineol, 14 (*E*)-β-caryophyllene, 22 (*E,E*)-α-farnesene, 24 δ-cadinene or τ-cadinene, 26 (*Z*)-α-bisabolene, 29 τ-cadinol. Measurements were done in at least five replicates and a representative GC-MS trace is shown.

Discussion

The *CsTPS* gene family is relatively small compared to *TPS* gene families in other higher plant species

The family of *TPSs* is a mid-sized gene family in plants (Chen et al., 2011). In this study, 27 *CsTPS* gene models were identified in the genome of *C. sativus* of which 19 seem to encode complete TPS proteins. These 19 *CsTPSs* form a relative small *TPS* family compared to other flowering plant species such as *A. thaliana* [40 putative TPS gene models; 32 with putative full length, (Aubourg et al., 2002)], *Solanum lycopersicum* [44; 29 (Chen et al., 2011)]; *Oryza sativa* [57; 34, (Chen et al., 2011)] and *Vitis vinifera* [152; 69, (Martin et al., 2010)]. The *TPS* family in apple consists of 55 gene models but only 10 of them are putatively functional (Nieuwenhuizen et al., 2013).

Structural and functional evolution of *CsTPSs*

CsTPS genes are located in several gene clusters within the cucumber genome. This clustered localization has been reported for most *TPS* gene families studied in various plant genomes. In Arabidopsis, 18 *AtTPS* genes are located in six small gene clusters ((Aubourg et al., 2002)). *TPS* gene clusters were also found in tomato, including a cluster containing nine genes and a second containing six genes on the same chromosome (Falara et al., 2011). Larger *TPS* gene clusters emerged in the grape genome where 85% of the grape *TPS* genes are organized in 13 clusters of which the largest cluster contains 20 complete TPSs and 25 pseudogenes (Martin et al., 2010). Consistent with the clustering of *TPS* genes in other plant species, more than 80% of the identified *CsTPS* genes are organized in four clusters located on three chromosomes. Only five (*CsTPS12-13, CsTPS23-24* and *CsTPS27*) of the 27 putative genes and pseudogenes are not located within a cluster. Clustering of metabolism-associated genes is relatively common and this may have several benefits. For example, the genes in these clusters are co-inherited and therefore stay together when they are part of the same biosynthetic pathway (Osbourn, 2010, Nutzmann and Osbourn, 2014, Chu et al., 2011, Takos and Rook, 2012). Furthermore, the genes in clusters could share similar regulation mechanisms such as chromatin modifications (Wegel et al., 2009). *TPS*s were reported to frequently co-localise with P450 genes in many species (Boutanaev et al., 2015). However, in cucumber only a single P450 gene and no members of other classes of genes, such as glycosyl transferases were found located within any of the TPS clusters. Just as reported for other species, *CsTPSs* located within the same cluster in the genome were assigned to similar clades in the phylogenetic tree. The subfamily TPS-a consists of proteins encoded by *CsTPS4-11* that are located within one cluster on chromosome II. Two gene clusters on chromosome III together encode the *CsTPSs* in group TPS-b. The TPS-g branch consists of three *CsTPSs* encoded by linear arrays of full length genes located on Chromosome I. This distribution

revealed that *CsTPS* genes within a genomic cluster are more homologous to each other than to the *CsTPS* genes outside that cluster and likely arose by tandem duplication. Evolutionary analysis of terpenoid biosynthesis related genes and supergene clusters of 17 genomes demonstrated that genes encoding TPSs are more enriched for tandem duplications than genes encoding enzymes involved in the upstream MVA pathway and IPP isomerases (Hofberger et al., 2015).

The amino acid sequences of CsTPSs were aligned to those extracted from the genomic sequences from *Cucumis melo* (muskmelon) and *Citrullus lanatus* (watermelon) (Fig. S1). The phylogenetic tree of *TPS*s from these three species showed that the *TPS* gene family continued to evolve after the differentiation of the three species. However, the number of genes present in each subclade, suggests that evolutionary events occurred more often in TPS-a and TPS-b but less in TPS-c, TPS-e/f, and TPS-g. TPS genes from *C. sativus* and *C. melo* are closer related to each other than to *C. lanatus*, which is in accordance with their evolutionary relation within the Cucurbitaceae (Renner et al., 2007).

As a consequence of the structural divergence of CsTPSs, their functions diverted from each other. As reported for many TPSs characterized in other plant species, CsTPSs can accept different substrates. At least in *in vitro* assays, most CsTPSs accepted both GPP and FPP, although with different efficiencies. Furthermore, 11 out of 15 CsTPSs that were tested in this study catalysed the formation of multiple terpenes from the same precursor, a common phenomenon in plant TPSs. For example, ten different monoterpenes were formed by a single TPS originating from Arabidopsis (Chen et al., 2004). Most of the characterized tomato TPSs catalysed the formation of more than one terpene structure (Falara et al., 2011). At the same time, a number of terpenes were synthesized by different CsTPSs. For example, linalool is the major product of CsTPS1, CsTPS2 and CsTPS3, and was also synthesized by most of the other CsTPSs. Most of the CsTPSs are able to convert GPP to acyclic monoterpenes including β-myrcene, (*E*)-β-ocimene and linalool, or convert FPP to acyclic sesquiterpenes including (*E*)-β-farnesene, (*E,E*)- α-farnesene and (*E*)-nerolidol. The formation of cyclic terpenes was catalysed by a limited number of CsTPSs including CsTPS10 that uniquely uses a pinyl cation to catalyse the formation of β-pinene, α-pinene, sabinene and α-phellandrene, which are not produced by other CsTPSs. Furthermore, cyclic sesquiterpenes including (*E*)-β-caryophyllene, α-humulene and τ-cadinol were produced by just a few CsTPSs.

The CsTPSs are predicted to be targeted to the cytosol, chloroplasts, mitochondria or the secretory pathway. Depending on the presence of terpenoid precursors that could differ in these compartments the product profile of CsTPSs *in planta* may differ from those *in vitro*. *Fragaria × ananassa* NEROLIDOL SYNTHASE 1 (FaNES1) was capable to generate both linalool and nerolidol when it was assayed *in vitro* (Aharoni et al., 2004). Targeting of FaNES1 to the plastids where the monoterpene precursor GPP is present, in transgenic Arabidopsis plants resulted in the production of linalool and its glycosylated and hydroxylated derivatives, and

only very low amounts of nerolidol (Aharoni et al., 2003). However, targeting of FaNES1 to the mitochondria where FPP is available resulted in emission of nerolidol 20 to 30 times higher than upon targeting to the plastids (Kappers et al., 2005). Co-overexpression of *FaNES1* with a gene encoding FPS1 (FPP SYNTHASE 1) which synthesizes FPP in mitochondria and a gene encoding HMGR1 (3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE 1) which enhances FPP availability in the cytosol in Arabidopsis resulted in increased emission of both linalool and nerolidol (Houshyani et al., 2013). Although in our study all active CsTPSs catalysed the formation of both monoterpenes and sesquiterpenes from GPP and FPP, respectively, the CsTPSs with plastid targeting peptides were predominantly effective in accepting GPP as substrate while CsTPSs without targeting peptide effectively accepted FPP. An exception is CsTPS19, that accepted both GPP and FPP efficiently to produce (*E*)-β-ocimene and (*E,E*)-α-farnesene, respectively, supporting that this is a genuine dual function TPS. The predicted subcellular localization of these CsTPSs coincides with the presence of the precursors which they effectively use. Thus, the enzymatic activity of the CsTPSs in combination with their subcellular localization together likely determine which terpene compounds are produced in cucumber.

Expression patterns of *CsTPS* genes display variation

TPS genes display different expression patterns across the different organs and this is consistent with the differences in expression of *TPS* genes across organs of Arabidopsis (Tholl and Lee, 2011) and tomato (Falara et al., 2011). Furthermore, the different expression patterns of *TPS* genes to some extent explains the differences in quantity and composition of terpenoids emitted from these organs and this may give hints to understand their biological roles within the plant. While the incomplete *CsTPS* genes generally have no or a quite low number of transcripts in any of the organs (with the exception of *CsTPS7)*, *CsTPS* genes with at least five exons (*CsTPS1-24*) were found to have detectable expression levels in at least one of the sequenced organs (except for *CsTPS17*). Expression patterns of *CsTPS* genes differ in male and female flowers. Seven *TPS* genes, *CsTPS1*, *CsTPS3*, *CsTPS5*, *CsTPS10*, *CsTPS19*, *CstPS21* and *CsTPS*22 are highly expressed in female flowers while only *CsTPS3*, *CsTPS5* and *CsTPS11* have a relatively high expression in male flowers. Although male flowers have higher absolute volatile emission, the volatile blend of female flowers encompasses more variation in terpenoids, including linalool, (*E*)-β-caryophyllene, (*E,E*)-α-farnesene, (*E*)-nerolidol, and τ-cadinol. The presence of these compounds in the headspace of female flowers is consistent with the higher expression levels of (*E,E*)-*α-FARNESENE SYNTHASE*, *CsTPS19*, (*E*)- β-*CARYOPHYLLENE SYNTHASE*, *CsTPS21* and τ-*CADINOL SYNTHASE*, *CsTPS22,* in flowers. As the emission of floral terpenes plays a key role in pollination in many plant species (Pichersky and Gershenzon, 2002), we hypothesize that the terpenoid-rich female flowers attract more pollinators. Female flowers have ovaries and are more costly to produce than male flowers and as there are much more male than female flowers on an

individual cucumber plant it is therefore essential for the plant to enhance the probability that pollinators also visit female flowers next to male flowers.

Expression of *CsTPS23* and *CsTPS24* that according to the phylogenetic analysis code for COPALYL DIPHOSPHATE SYNTHASE and ENT-KAURENE SYNTHASE, respectively, differed in fertilized and unfertilized ovaries seven days after flowering. As both these enzymes are known to participate in gibberellin biosynthesis (Yamaguchi et al, 2008) and gibberellins are suggested to have an important role in fruit development and the regulation of parthenocarpy (Talon et al, 1992; Fos et al, 2001), it would be interesting to study to what extent these CsTPSs contribute to this.

Potential roles of CsTPSs in spider-mite induced volatile formation

The volatile blend of unchallenged cucumber leaves contained few terpenoids in low amounts including limonene, (*E*)-β-ocimene and linalool which coincides with expression of *CsTPS1*, *CsTPS2*, *CsTPS3* and *CsTPS5* in unchallenged leaves. Limonene formation can be catalysed by CsTPS1 and CsTPS3, (*E*)-β-ocimene by CsTPS1 and CsTPS5, and linalool by CsTPS1, CsTPS2 and CsTPS3.

Upon spider-mite infestation, the expression of *CsTPS2-5, CsTPS9* and *CsTPS19* increased suggesting a role of these genes in spider-mite induced volatile emission. Previous studies (Takabayashi et al., 1994, Bouwmeester et al., 1999, Agrawal et al., 2002, Mercke et al., 2004, Kappers et al., 2010, Kappers et al., 2011) documented the volatile blends emitted by different accessions of spider-mite infested cucumber. Also in spider-mite infested leaves of *C. sativus* Chinese Long, with bitter foliage, the main components in the induced blend are terpenoids, including (*E*)-β-ocimene, (*E*)-DMNT [(*E*)-4,8-dimethyl-1,3,7-nonatriene], (*E,E*)-α-farnesene and (*E,E*)-TMTT [(*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene]. Furthermore, linalool was emitted in relatively higher amounts from mite-infested leaves compared to non-infested leaves, and monoterpenes including α-pinene, α-phellandrene and sabinene, and sesquiterpenes (*E*)-β-farnesene and α-humulene were emitted in small amounts by spider-mite infested leaves and not by non-infested leaves. Previously, the induction of *(E)*-β--*OCIMENE/(E,E)-α-FARNESENE SYNTHASE* (in this study *CsTPS19*) in spider-mite infested cucumber leaves was demonstrated (Mercke et al., 2004). Transcripts of genes encoding monoterpene synthases CsTPS1-3 (LINALOOL SYNTHASE), CsTPS5, CsTPS9 [(*E*)-β-OCIMENE SYNTHASE] and sesquiterpene synthase CsTPS19 [(*E*)-β-OCIMENE/(*E,E*)-α-FARNESENE SYNTHASE] increased upon infestation of spider mites within the first three days. All these genes except *CsTPS1* were still upregulated compared to non-infested plants nine days after infestation and are suggested to be involved in the production of the herbivore-induced volatile blend of cucumber. Considering the multiple minor products that are produced by

these CsTPSs beside their major products, induction of these genes enables the plants to produce a wide spectrum of volatiles in response to herbivory.

Different transcriptional regulation of these *CsTPS* genes could result in complicated changes in compositions and quantity of terpenoid volatiles emitted by cucumber plants upon different environmental stimuli. Although we did not compare the blends of volatiles emitted by cucumber in response to herbivory by different herbivores, it is reported that plants can emit different blends of terpenoid volatiles upon infestation by different herbivores. For instance, lima bean plants emitted different blends of volatiles including different amounts of (*E,E*)-TMTT upon herbivory by *Spodoptera exigua* or *T. urticae* and consequently, the predatory mite *P. persimilis* was more attracted to plants infested by its prey, *T. urticae* (De Boer et al., 2004). Moreover, when lima bean and cucumber plants were infested by *S. exigua* and *T. urticae* separately or together, the plants emitted different amounts of volatile compounds including several terpenes and the plants treated with multiple herbivore species were more attractive to predatory mites than the plants damaged by only a single herbivore species (de Boer et al., 2008). The diversity of terpenoids produced by CsTPSs and the variation in the regulation of expression of the corresponding genes gives the cucumber plant the ability to emit different blends of terpenoids, if required for the interaction with its environment. Moreover, a number of the *CsTPS* genes differ in their expression in both cucumber accessions included in the present study, either in unchallenged leaves or in response to spider-mite infestation (e.g. *CsTPS2*). This reveals the presence of genetic diversity such that different accessions can have different blends of volatile terpenoids, which matches the observation of variation in volatile blends emitted by different cucumber accessions in response to infestation by spider mites and hence differences in the attractiveness towards their natural enemies (Kappers et al., 2010, Kappers et al., 2011).

Most of the terpenoids emitted by non-infested or infested leaves or other organs of cucumber correlated well with the product profiles of CsTPSs and the expression of the corresponding genes. An exception is the increased emission of α-pinene, α-phellandrene and sabinene by cucumber leaves during herbivory of spider mites, while the gene encoding the most likely corresponding terpene synthase (CsTPS10) was not upregulated as result of infestation. An explanation could be that other genes, in the pathway upstream of the TPS are also involved in the emission of terpenoids. In cucumber, genes encoding a DXP-synthase and two GPP synthases in the MEP/DOXP pathway which is responsible for the biosynthesis of terpenoid precursors in the plastids were found upregulated and a gene encoding HMGR1 in the mevalonate pathway which produces precursors for sesquiterpene and triterpene biosynthesis in the cytosol was downregulated during three days of spider-mite herbivory (Chapter 2), indicating that in the cells of infested plants precursors for monoterpene and diterpene synthases could be enhanced upon herbivory. This could have implications for the product formation by TPSs that are constitutively expressed. Hence, the terpenoid

metabolite profile will be determined by TPSs that are induced upon herbivory as well as those that are constitutively expressed.

Abundant amounts of *(E)*-DMNT and (*E,E*)-TMTT are present in the volatile blend emitted by cucumber leaves infested with spider mites (Kappers et al., 2010, Kappers et al., 2011), and most of the CsTPSs catalysed the formation of (*E*)-nerolidol, the precursor for (*E*)-DMNT *in vitro* while only CsTPS9 and CsTPS24 were able to catalyse the formation of (*E,E*)-geranyl linalool, the precursor for (*E,E*)-TMTT. The C15 (*E*)-nerolidol is never detected in the headspace of mite-infested leaves of cucumber (unpublished results I.F. Kappers), indicating an efficient conversion of (*E*)-nerolidol into the corresponding C11 homoterpene, (*E*)-DMNT. In Arabidopsis, a cytochrome P450 monooxygenase (CYP82G1, At3g25180) catabolizes the C20-terpene (*E,E*)-geranyl linalool to the insect-inducible C16-homoterpene (*E,E*)-TMTT (Lee et al., 2010) and it has been suggested that a similar enzyme is responsible for the conversion of (*E*)-nerolidol into (*E*)-DMNT (Kappers et al., 2005). A *P450* gene is located near the *TPS* cluster on chromosome II, that includes *CsTPS4-11*, however a BLAST search indicates that this gene belongs to the 78A group of cytochrome P450 genes, not known to be involved in terpenoid biosynthesis. Profiling gene expression of cucumber leaves infested by spider mites (Chapter 2), revealed transcripts of seven cytochrome P450 genes that were differentially expressed including four genes upregulated and three downregulated. Although these genes do not co-localise with the *CsTPS* genes, they may be candidates for the oxidation of CsTPSs products to form terpenoid compounds such as (*E*)-DMNT and (*E,E*)-TMTT. Additional experiments are needed to study whether one of these P450 enzymes could convert the products of the CsTPSs into other compounds and hence enlarge the diversity in terpenoids.

Conclusion

In conclusion, we identified the cucumber (*C. sativus*) *TERPENE SYNTHASE (CsTPS)* gene family from the sequenced genome and characterized the enzymatic activities of the members with a bonafide TPS structure. We identified the *CsTPS* genes that contribute to the terpene volatiles in cucumber, for example as induced by spider-mite herbivory and revealed the variation in the expression of the *CsTPS* genes as well as the different profiles of terpene volatiles among different cucumber organs.

Material and Methods

Identification of *CsTPS* genes Database searches and re-annotation

The *Cucumis sativus* genomic database (version 2.0; Li et al., 2011) was searched for *CsTPS* gene candidates using InterProScan (Zdobnov and Apweiler, 2001) to the default protein dataset. Proteins with at least one domain of either IPR001906, IPR005630, IPR008930 or IPR008949 were assigned to be *CsTPS* candidates. Genomics regions containing these genes and their flanking 4 Kb sequence were extracted and re-annotated and confirmed by Fgenesh and Genewise according to the structure of previously reported TPS proteins (Chen et al., 2011). Signal peptide prediction was done using the TargetP 1.1 server (Emanuelsson et al., 1999). Sequences will be deposited in Gen-bank.

Multiple-sequence alignment and phylogeny construction

The ClustalW (Thompson et al., 1994) and MUSCLE (Edgar, 2004) algorithms were used to make an amino acid alignment of all full length CsTPS enzymes. Phylogenetic trees were constructed using the maximum likelihood method in MEGA5 (Tamura et al., 2011). Parameters were set as: Substitution model was set as “Poisson model”, Rates and Patterns was set as “Uniform rates”, Data Subset to Use was set as “Partial deletion”. The phylogenetic relation of the different sub-clades was identified according to the reported *TPS* family in Arabidopsis.

Plants and insects

Cucumis sativus plants with bitter foliage (‘Chinese long’ inbred line 9930 from IVF) were grown in potting soil (Lentse potgrond) in a greenhouse maintained for 16 h in the light at 20 ± 1 °C and 8 h in the dark at 18 ± 1 °C and a relative humidity of 60 ± 5%. Plants were grown for 5 to 6 weeks until they had multiple flowers and first fruits. For herbivory-induction experiments plants were grown for 3 weeks until 4 or 5 true leaves were developed. For volatile collection of roots, plants were grown in Rockwool planting cubes (Grodan, Roermond, The Netherlands) in an aeroponic system were nutrients (¼ Hoagland) and water are sprayed onto the roots in mist form using a pump ensuring enough oxygen to the roots. Two-spotted spider mites (*Tetranychus urticae*) were reared on lima bean plants reared in a greenhouse (16h light, 21 ± 1 °C / 8h dark, 18 ± 1 °C) The second fully expanded cucumber leaf was infested with 50 adult spider mites when the plant had 4 or 5 true leaves in total. Infested cucumber leaves were sampled in liquid nitrogen at different time points and stored at -80 °C for later experiments. All tissues were harvested in triplicate from independent plants.

RNA isolation and qPCR

Total RNA was isolated using TriPure (Roche, Mannheim, Germany) and purified using the RNeasy Mini Kit (Qiagen, USA) from different cucumber tissues according to the manufacturer's instructions. RNA was purified using the RNeasy Mini Kit (Qiagen, USA) and treated by DNase. RNA quality and quantity was checked by Nanodrop 1000 (Thermo scientific, USA). One µg high quality RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) at 25 °C,5min; 42 °C, 30 min; 85 °C, 5 min with an oligo-dT primer to produce single-stranded cDNA for qPCR and full length cDNA cloning. For each of the samples RNA was extracted and reverse transcribed in duplicate.

For qPCR, cDNA was diluted 10-fold using fresh MQ water and 2 µL was used as template in a 20 µl reaction system. The iQ^{tm} SYBR$^{@}$ Green Supermix (Contains SYBR$^{@}$ GreenI I dye, iTaqTM DNA polymerase, dNTPs and fluorescein) and qPCR machine (BioRad iQ 5 RTPCR QPCR) from Bio-Rad (Hercules, CA, USA) were used. The program was set as below: 95 °C for 3 min, 40 cycles of 95 °C for 10 and 57 °C for 30 min. The amplified products were assessed by a final dissociation step to insure the amplification quality. For each *CsTPS* gene a specific primer combination was designed to amplify a region of 80–120 bp (Table S1.) Primer specificity was confirmed by aligning the primers to the cucumber genome using BLAST (Altschul et al., 1997). Three biological replicates were analysed in triplicates (technical replicates). Relative expression levels were calculated by normalizing to the expression levels of cucumber β-*Actin* (Csa6M484600) using ddCt method in software Bio-Rad iQ5 (Bio-Rad, Hercules, CA, USA).

Full-length cDNA cloning

To obtain full length clones for each of the predicted *CsTPS* genes, specific primers were designed to clone the open reading frames (Table S2). Restriction sites of BamHI and SalI were attached to the front and end of each of the targeting fragments respectively. The full length cDNA sequences of the *CsTPS* genes were amplified and cloned into a TA vector pJET and subsequently sequenced. For functional characterization the full length genes were sub-cloned into the expression vector pACYCDuet under the control of an IPTG inducible T7 promoter and transformed into the *Escherichia coli* strain BL21DE3 for heterologous expression.

Heterologous expression and enzyme activity assay

Bacteria containing *CsTPS* genes in vector pACYCDuet were cultured in 5 ml LB solution supplemented with 1% glucose and 34 µg/ml chloramphenicol at 250 rpm and 37 °C

overnight. Five hundred µl of this starter culture was diluted in 50 ml 2XYT (1.6% Bacto, 1% Bacto Yeast Extract, 0.5 NaCl, PH=7.0) added with 34 $\mu g.ml^{-1}$ chloramphenicol and grown until OD_{600} 0.6-0.8. Production of heterologous protein was induced by adding 50 µl 1M IPTG and the cultures were subsequently incubated at 18°C overnight. Bacterial cells were collected by centrifugation and re-suspended in ice-cold extraction buffer (50 mM Tris, PH7.5, 300 mM NaCl, 1.4 Mm β-mercapto ethanol). The cells were disrupted by sonication (5 x 10 s) with a sonicator (Soniprep 150, MSE, UK) on ice and subsequently centrifuged (14,000 *g*) to collect the clarified crude protein extract.

To determine the catalytic activity of the different terpene synthases, 150 µl of the crude enzyme solution and 1340 µl assay buffer (15mM MOPSO, 12.5% (w/v) glycerol, 1 mM ascorbic acid, 100 µl Tween-20, 1mM $MgCl_2$, 2 mM DTT) were incubated with 10 µM (*E,E*)-FPP or (*E*)-GPP. For specific TPS genes also (*Z,Z*)-FPP, NPP and (*E,E,E*)-GGPP were used as substrate. Based on the method described previously (David and Sandra, 2007), a 10 mm polydimethylsiloxane (PDMS film thickness 1 mm) stir bar (Gerstel, Mülheim a/d Ruhr, Germany) was enclosed in each assay vial for 60 min incubation at 30 °C with 250 rpm shaking. Subsequently, the stir bar was removed from the assay tube, briefly rinsed in water, dried under a stream of nitrogen and stored in a closed vial under nitrogen until analysis. As a negative control, raw protein extracts from *E. coli* expressing the empty pACYCDuet vector with substrates (FPP, GPP or GGPP) were incubated as described above.

The PDMS stir bars were enclosed in a glass liner and volatiles were desorbed and analysed in a Thermo Trace GC Ultra connected to a Thermo Trace DSQ quadrupole mass spectrometer as previously described (Kappers et al., 2011). In brief, volatiles were released by heating to 250 °C for 3 min with a helium flow of 30 ml/min. Released volatiles were transferred to and condensed in a cold trap at -10 °C within the Thermo Desorption Unit. Then the cold trap was heated to 300 °C and volatiles were injected in splitless mode into an analytical column (Restek, RTX 5MS, 30 m 0.25 mm i.d.,1.0 µm df). The initial temperature of the GC was 40°C and lasted 3.5 min followed by a temperature increase with a linear gradient of 10°C/min to 280°C and held for 2.5 min with a helium flow of approximately 1 ml/min. The ion source of the DSQ quadrupole mass spectrometer was directly coupled with the analytical column and was operated in the 70 eV EI ionization mode. Mass 45 to 400 *m/z* were scanned. Automatic desorption of the Tenax traps (UltrA™, Markes international Ltd, UK) was done with an auto sampler. PDMS stir bars in between the measurements were cleaned by heating them to 310°C for 40 min with a helium flow using TC-20 Multi-tube conditioning unit (Markes International Ltd, UK). Compound identification was performed using Wiley (www.sisweb.com/software/wiley-ffnsc.htm), Adams mass spectra databases and retention indices (Adams, 2007); NIST05 (http://chemdata.nist.gov/), and an in-house data base in which authentic standards from Sigma were used to identify compounds. Essential oil of basil (*Ocimum basilicum*) was used to characterize τ-cadinol. For each TPS enzyme and substrate combination, assays were repeated at least once but most often three

times. For all replicates, the major products were similar and in the same order of relative magnitude.

Cucumber volatiles collection and analysis

To investigate the volatile emission of different cucumber organs, we used PET bags (roasting bags, Toppit, Germany) adapted with an inlet and outlet to enclose individual organs. Individual leaves, male and female flowers, and roots were enclosed using tie-wraps and volatiles were collected from 11.00 until 16.00 (5 hrs). Tenax liners (20/35-mesh, Alltech) were used to filter the incoming air (flow rate 150 ml. min^{-1}) and collect volatiles from the out coming air (flow rate 100 $ml.min^{-1}$). Volatile emissions were collected for each organ from five independent plants. After collection, tenax liners were dry-purged for 20 min under a stream of nitrogen and subsequently analysed on a thermo-desorption GC-MS (Thermo Trace GC Ultra, Thermo Fisher Scientific, Waltham, MA, USA) connected to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, MA, USA) quadrupole mass spectrometer as described above.

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Supplementary data

Table S1. The primers for *CsTPS*s used in qPCR.

Name	Gene ID	Forward	Reverse
CsTPS1	*Csa1M066550*	GCATGGTTGGAACCCTATCCATTGC	TGGCCAGAAGCAAACCATTCTGCT
CsTPS2	*Csa1M066560*	TCTCCTGCAACCATTCTGCGTCT	TGCTTTCGAGTTGTGTCGATCGAA
CsTPS3	*Csa1M068570*	TGCTAGGCCAACAAATTACCGACG	ACCCATCTCGCCCCTCTTGCT
CsTPS4	*Csa2M298300*	AGCCATCATTGGCGGCATGT	GGTCAATGAATCGCCCATCTCCGT
CsTPS5	*Csa2M298310*	ACACCCGTACCGGATGAACAAAACA	ACGAGCATTTTTCTCTGAACTGCCT
CsTPS7	*Csa2M299330*	TGTCTCCAGATGGGATATCGGTGCG	CATTGATGGTGTTGTGGAGGGCA
CsTPS9	*Csa2M299870*	AACCCGACTCAAATCCAGTGTTGTT	TCCTGTGCTCTTCCACCACCTG
CsTPS14	*Csa3M039850*	TGGGAATTGCTGCTTCACAAGAGGC	AGCCAGTGGAATAGTTGGGAGGCT
CsTPS19	*Csa3M095040*	AGCCTCCGCTGTTATTTGTAGGCT	GCAGAGGCCACATGACCTCGC
CsTPS21	*Csa3M097040*	TAGCTGAAATCTGCAGGTGGTGGA	ACACTCCACAATTCGATCCCTCGC
CsTPS22	*Csa3M097540*	TGGACGACATCGCTTCCCACA	TCCTTCCATGCATCAACCACTTCCT
Actin	*Csa6M484600*	GCCGAGGATATTCAGCCCCTCGT	CCAGTATGCCGGGGACGACCA

Table S2. Primers for isolation of cDNA of *CsTPS*s.

Gene name	Gene ID	Forward	Reverse
CsTPS1	*Csa1M066550*	AGGATCCGATGGCTACAGCATCTGCCATG	CGTCGACTTAAATTTGTACACTTTCATAGAGCAC
CsTPS2	*Csa1M066560*	AGGATCCGATGGCTGAAACAAAATTCCCAG	CGTCGACTTACATGTGCACTTTTTCGTACAAC
CsTPS3	*Csa1M068570*	AGGATCCGATGGCTTTTTCTGCATTTGC	AGTCGACTTACATTTCCACGCCATCC
CsTPS4	*Csa2M298300*	AGGATCCGATGTCTCTCGTTTACTTTCGTCTTC	GGTCGACTTAACGAGTACTGATAGGATGAACAAG
CsTPS5	*Csa2M298310*	AGGATCCGATGGCTCTTCTCCACCTCCC	GGTCGACTTAATGAATGATAATAGGTGTTACAAGAAG
CsTPS9	*Csa2M299870*	CGGATCCGATGGCTATTCTTCATCATCCTCTT	CGTCGACTTAAGCAGGCTTGATAAATAAGGAT
CsTPS10	*Csa2M299890*	TGGATCCGATGGCTCTTCACCAATTTCCC	CGTCGACTTAAATAACCTTAGGCAAGTTTTCAC
CsTPS11	*Csa2M299920*	CGGATCCGATGGCTCATCTTCATCAAATTT	GGTCGACTTATTCACTATATGAACAATGAATGG
CsTPS12	*Csa2M427840*	GGGATCCGATGTCATTTCAAATAACTCCATCAG	TGTCGACTCAAATTGGCACAGGCTCT
CsTPS13	*Csa3M021130*	GGGATCCGATGAGTAGTGTATCATCCATGAATGG	CGTCGACTTAAATTGGTATTGGATCGAGAACT
CsTPS14	*Csa3M039850*	CGGATCCGATGTCATTTGAAGATAAAGGTTCACTC	CGTCGACTCATATTGGTATGGGGTCTATAAGC
CsTPS15	*Csa3M040850*	GGGATCCGATGGAAAGAAAAATGTCATTTGAAG	AGTCGACTCAAATTGGCATGGTGAGCG
CsTPS17	*Csa3M041370*	CCTGCAGTTATGGCAAAGGGTCTATAAGCAT	CGAGCTCGATGTCATTTCAAATACTTGAATGTGAT
CsTPS18	*Csa3M042380*	GGGATCCGATGGCATATGAAGAAATTATAGCAG	TGTCGACTCAAGTTGGAACAGGGTGAGT
CsTPS19	*Csa3M095040*	CGGATCCGATGAGTTCAAACGTATCAGCAATTC	AGTCGACTTAGAGTGGTACAGAATCAACAAGC
CsTPS21	*Csa3M097040*	CGGATCCGATGTCTTCTCATTTTCCTGCTTC	GGTCGACTTACAAATGCAATGGATCAATAAG
CsTPS22	*Csa3M097540*	GGGATCCGATGAAAACCAATGATATTTCTGATG	AGTCGACTTAACAAGGCAGTGGGTCAAC
CsTPS23	*Csa6M410650*	AGGATCCGATGTCTCTTTCCACCCTCATCC	CCTGCAGTCAGAACACTTTTTCAAATAGGACC
CsTPS24	*Csa7M239640*	AGGATCCGATGAATCTTTCCCGACCCAC	GGTCGACTCATTTGTTCAAAAGTTCATCCAG

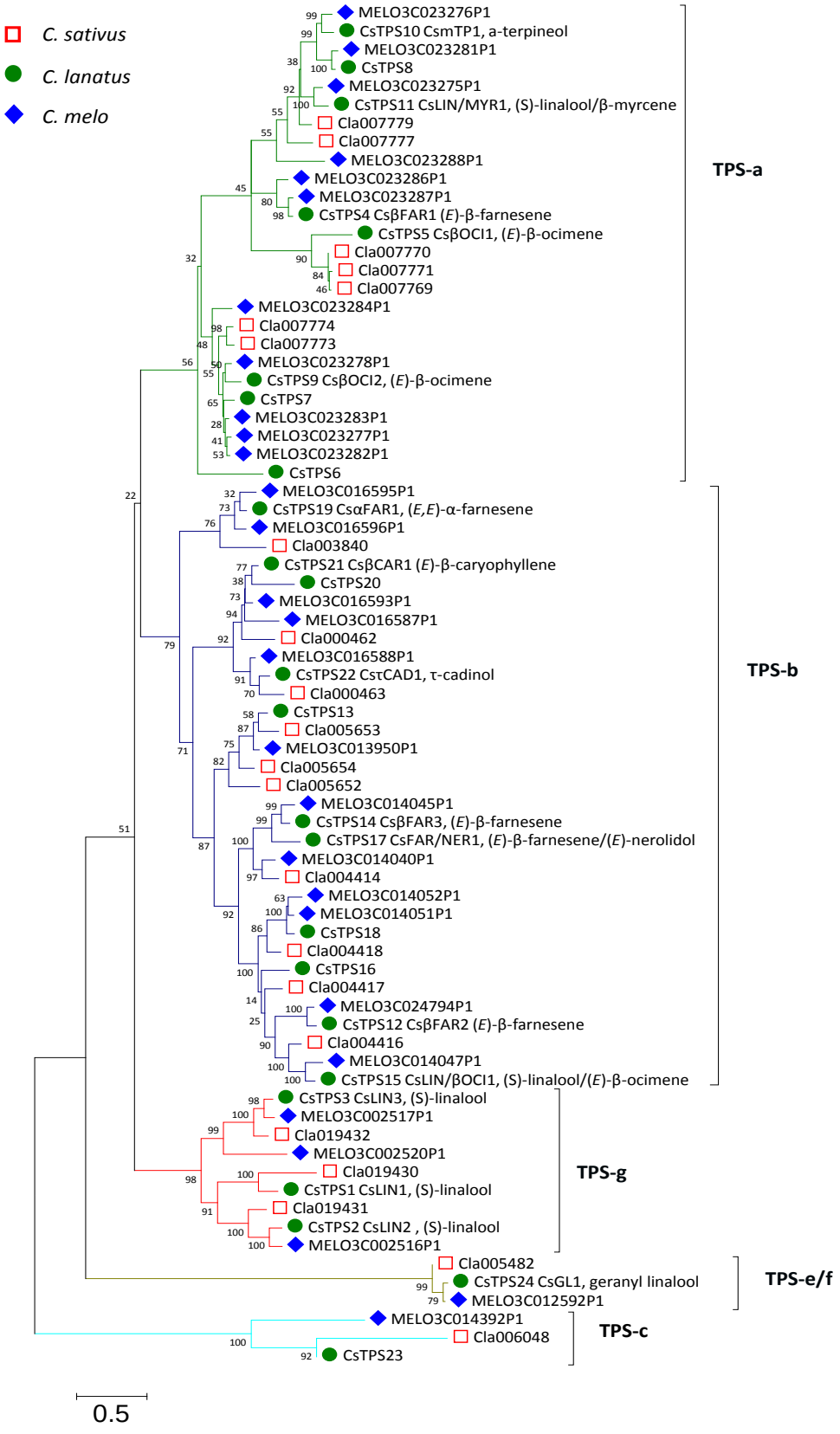

Fig. S1. Phylogenetic tree of putative full length TPS proteins from *Cucumis sativus*, *Citrullus lanatus* and *Cucumis melo.* The name of CsTPSs and the major products they can form are indicated.

Chapter 5

Promoters of cucumber defence-related genes respond to herbivory, mechanical wounding and jasmonic acid in a light-dependent rhythm in transgenic Arabidopsis plants with a GUS/LUC fused reporter system

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Abstract

Plants have complex defence systems to survive from the threats of herbivores. For example, upon infestation plants regulate the expression of whole cascades of defence genes. We previously identified genes encoding two terpene synthases (*CsTPS9* and *CsTPS19*) and a lipoxygenase in cucumber (*CsLOX*) that were induced upon two-spotted spider-mite (*Tetranychus urticae*) infestation. Here, we characterize the spatial and temporal dynamics of the promoter activity of these inducible defence genes *in planta*. The promoters of *CsTPS9*, *CsTPS19* and *CsLOX* were fused to *GLUCURONIDASE* (*GUS)/FIREFLY LUCIFERASE (LUC)* and introduced into Arabidopsis after which their response to herbivory by different species, hormonal stimuli and wounding was investigated. LUC activity in transgenic plants was below the threshold in non-challenged plants but increased rapidly when cell-content feeding spider mites and thrips (*Frankliniella occidentalis*) were introduced. In contrast, phloem-sucking aphids (*Myzus persicae*) did not induce promoter and hence LUC activity. The promoters of the two terpene synthase genes were also responsive to mechanical damage and jasmonic acid but not to salicylic acid. Under a 12h light/12h dark cycle LUC activity of transgenic P_{CsTPS9}::*GUS/LUC* and P_{CsLOX}::*GUS/LUC* reporter plants peaked during the dark period and decreased during the light period. $P_{CsTPS19}$::*GUS/LUC* activity increased with progressing infestation but no obvious rhythm was observed in the first three days. Under continuous light, both P_{CsTPS9}*::GUS/LUC* and $P_{CsTPS19}$:: *GUS/LUC*, but not P_{CsLOX}*::GUS/LUC* reporter plants exhibited a circadian LUC activity rhythm, while under continuous darkness the circadian rhythm diminished. Emission of herbivory-induced volatiles by spider-mite infested cucumber plants was highest during the light period. These results indicate that both P_{CsTPS} and P_{CsLOX} are stimulated by herbivory and regulated by light and possibly the circadian clock, which together results in terpenoid volatile release in response to herbivory by spider mites, particularly during the day.

Keywords: Inducible promoter, Circadian rhythm, Terpene synthase, Lipoxygenase, GUS/LUC reporter, Arabidopsis, Cucumber, Spider mites.

Introduction

Plants can quickly react to stresses imposed by their environment, for example upon attack by herbivores. This so-called inducible defence is either not present constitutively, or at a low level, under non-stressed conditions, but is initiated upon herbivory. Plants can detect feeding by herbivores and can even discriminate these herbivores depending on their feeding habit. Some herbivores (such as caterpillars) inflict significant physical damage by biting off and ingesting parts of the tissue, while others (such as aphids) cause only little tissue damage by sucking the sap from the phloem by inserting their stylets into the plant sieve element. Other herbivores inflict more moderate damage, such as spider mites and thrips that pierce plant tissues and feed on mesophyll cell contents. In addition to mechanical wounding, the infested plant gets into contact with cues from the herbivores within their oral secretion and both the mechanical and oral secretion cues trigger a cascade of reactions including early signalling such as a Ca^{2+} flux and the burst of reactive oxygen species followed by changes in the content of phytohormones such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) that are well-known plant hormones involved in the defence response (Wu and Baldwin, 2010). The synthesis, perception and cross-talk of these hormones, the transcription factors involved and their target genes together constitute a complicated signal-transduction network. Through this network, plants can re-program their transcriptome, activate defence-related transcription factors, regulate expression of primary or secondary metabolite biosynthesis genes, and subsequently reshape the defensive state of the plant. It is reported that the JA-signalling pathway is usually induced by chewing herbivores such as caterpillars (McCloud and Baldwin, 1997) and cell-content feeding herbivores such as spider mites and thrips (Dicke et al., 1999, Ament et al., 2004, Abe et al., 2009), while the SA-signalling pathway is induced by sucking herbivores such as aphids (Moran and Thompson, 2001).

Signal transduction in regulatory networks causes changes in gene expression, for example of genes involved in the biosynthesis of metabolites, and this may result in improved plant defence against herbivores. Toxic metabolites in plants may increase upon feeding by herbivores, such as glucosinolates in the Brassicaceae family, and repel or even kill herbivores (Hopkins et al., 2009). Furthermore, plants can emit a wide range of volatile organic compounds (VOCs) which may be attractive to natural enemies of the herbivores and defend the plant indirectly (Dicke and Sabelis, 1988). Among these herbivore-inducible volatiles, terpenoids and green-leaf volatiles represent the major classes of compounds (Van Poecke et al., 2001, Kappers et al., 2010). Biosynthesis of terpenoids and green-leaf volatiles is catalysed by terpene synthases (TPSs) and lipoxygenases (LOXs), respectively, and in agreement with the increase in VOCs emitted by plants in response to herbivory, genes encoding TPSs and LOXs were up-regulated in multiple plant species such as Arabidopsis (Bell et al., 1995, Huang et al., 2010, Chauvin et al., 2013), rice (Zhuang et al., 2012, Duan et al., 2014), tomato (Heitz et al., 1997) and cucumber (Mercke et al., 2004). Analysis of the

dynamics of the expression of *TPS* and *LOX* genes may reveal the dynamics of the response of plants to herbivory.

Plant defence can also be influenced by abiotic factors, especially light and day/night changes. In Arabidopsis, the expression of more than 40% of the genes induced by mechanical damage (MD) peaks at dusk and over 80% of the genes supressed by mechanical damage peak at dawn (Walley et al., 2007). Arabidopsis plants that were entrained under a similar light/dark rhythm with the cabbage looper *Trichoplusia ni* which has rhythmic feeding behaviour, had increased resistance to this herbivore, while plants grown under opposite day/night rhythm as the insect became more susceptible (Goodspeed et al., 2012). Both the circadian clock and jasmonates were shown to be essential in maintaining this rhythmic defence (Goodspeed et al., 2012). Application of methyl jasmonate to Norway spruce (*Picea abies*) induced the emission of volatiles over a period of seven days, with higher emission during the day and lower emission during the night (Martin et al., 2003). Kidney bean (*Phaseolus vulgaris)* plants infested by larvae of the leaf miner fly *Liriomyza huidobrensis* emitted green-leaf volatiles and terpenes in a clear day/night rhythm which peaked at the end of the day (Sufang et al., 2013). In *Artemisia annua,* expression of *QH6,* encoding for (-)-α-PINENE/(-)-β-PINENE SYNTHASE, was highest nine hours after the onset of the light period and lowest at nine hours after the end of the light period. In constant light the circadian oscillation of *QH6* expression was accelerated and in constant darkness it slowed down (Lu et al., 2002).

In the signal-transduction network of plant hormones, hormone–related transcription factors and their target promoters are an important part of the signal cascade. Promoter regions of the target genes contain motifs which can be recognized by these transcription factors. Mutation and deletion analysis of the jasmonate- and elicitor-responsive element (JERE) in the promoter of *STRICTOSIDINE SYNTHASE* (*STR*) from *Catharanthus roseus* identified a GCC-box-like sequence (Menke et al., 1999). A similar, although slightly different GCC motif was identified in the promoter of *PLANT DEFENSIN 1.2* (*PDF1.2*) in Arabidopsis which was responsive to a combination of JA and ET. The G-box (CACGTG) functions in promoters of multiple JA-responsive genes such as *PROTEINASE INHIBITOR 2* (*PIN2*) from potato (Kim et al., 1992), *VEGETATIVE STORAGE PROTEIN B* (*VSPB*) from soybean (Mason et al., 1993) and *OCTADECANOID-DERIVATIVE RESPONSIVE AP2-DOMAIN* (*ORCA3*) from *C. roseus* (Endt et al., 2007). Luciferase activity driven by the promoter of the *A. annua QH6* with a mutated G-box showed a rhythm lacking one peak in the early morning which was present when an intact G-box was present (Zhou et al., 2015). In addition, motifs such as jasmonate-responsive elements JASE1 (CGTCAATGAA) and JASE2 (CATACGTCGTCAA), L/AC-1 or H-box-like motifs [CCTACC$(N)_7$CT] are involved in JA-mediated regulation of gene expression (Loake et al., 1992, He and Gan, 2001). A number of *cis*-acting regulatory elements involved in SA responsiveness have also been reported. The SA-responsive *cis*-acting element (TGACG) acts as a transcriptional enhancer in response to SA (Sa et al., 2003) The W-box [(T)TGAC(C/T)] is important for expression of SA-responsive genes such as *S GENE*

FAMILY RECEPTOR 2 [*SFR2,* Rocher et al. (2005)] and *ACIDIC CHITINASE* [*VCH3,* Li et al. (2006)]. Various *cis*-acting elements were also identified in the promoter of genes of which the expression is responsive to light. For example, motifs CTCCAACAAACCCCTTC and ATTCTCACCTACCA in the promoter region of *PHENYLALANINE AMMONIALYASE*-1 (*PAL-1*) in parsley were found to be essential for the induction of *PAL-1* by UV light (Lois et al., 1989). The I-box (GATAAGA) and G-box (CACGTG) are important for regulatory functioning of Conserved Modular Array 5, which is the shortest light-responsive unit in the promoter of *RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE* (*RBCS8B*) from *Nicotiana plumbaginifolia* (Martinez-Hernandez et al., 2002, Lopez-Ochoa et al., 2007). A number of motifs were also identified in the promoter regions of genes that are responsive to stresses. The Rapid Stress Response Element (RSRE) containing six-nucleotide repeats (CGCGTT) was identified by analysing the promoters of genes with increased transcription for overrepresented motifs immediately after wounding (Walley et al., 2007). Four tandem repeats of RSRE were shown to be sufficient to confer a response in transgenic Arabidopsis to different stresses including wounding, oligouronides, insect regurgitant, feeding by cabbage loopers, infection by the necrotrophic fungus *Botrytis cinerea* and cold stress (Walley et al., 2007).

Induction of plant defences can be initiated at the site that is infested (local response) and in distal leaves which are not infested (systemic response). The undamaged leaves of corn seedlings injured by caterpillars emitted significantly more terpenoids than leaves of uninjured plants and were more attractive to the parasitoid *Cotesia marginiventris* (Turlings and Tumlinson, 1992). Two to three days of infestation by *Spodoptera exigua* on lower leaves of cotton plants induced the release of volatiles from the upper, undamaged, leaves of the same plant (Rose et al., 1996). Non-volatile secondary metabolites involved in direct defence can also be induced systemically, such as increased accumulation of cucurbitacin C in the first systemic leaf after the introduction of spider mites on the cotyledons of cucumber (Agrawal et al., 1999).

Previously, we have analysed gene expression in cucumber foliage in response to infestation by the two-spotted spider mite (*Tetranychus urticae*) using RNA-seq or micro-array analysis [Chapters 2 and 4, Mercke et al. (2004)] and found that expression of (*E*)-β-*OCIMENE SYNTHASE*, (*CsTPS9*), (*E*)-β-*OCIMENE*/(*E,E*)-α-*FARNESENE SYNTHASE* (*CsTPS19*) and a lipoxygenase (*CsLOX*) increased within the first three days of infestation. In the present study, we performed promoter-reporter analysis for *CsTPS9*, *CsTPS19* and *CsLOX in planta* using a double *FIREFLY LUCIFERASE (LUC)/β-GLUCURONIDASE (GUS)* reporter and studied their activity under several different treatments, such as herbivory and hormone treatment.

Results

Selection of spider-mite inducible genes for promoter analysis

Transcripts of *CsTPS9* and *CsTPS19* were present in low amounts in non-infested cucumber plants but progressively increased when spider mites were introduced (Fig. 1A, Chapters 2 and 4). The function of *CsTPS9* and *CsTPS19* was identified previously [Chapter 4, Mercke et al. (2004)] and both enzymes accept geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), the precursors of mono- and sesquiterpenes, respectively. CsTPS19 is considered to be a sesquiterpene synthase *in planta* as it lacks a targeting signal peptide and in *in vitro* assays (*E,E*)-α-farnesene, (*E*)-β-farnesene and (*E*)-nerolidol were detected, but no aspecific farnesol, after incubation with FPP, indicating high efficiency in the conversion of this substrate, while incubation with GPP resulted in formation of a substantial amount of aspecific geraniol next to a variety of specific monoterpenes (Fig. 1B, Chapter 4).

As CsTPS9 possesses a signal peptide putatively targeting the enzyme to the chloroplast, we considered it a monoterpene synthase predominantly making (*E*)-β-ocimene from GPP and β-myrcene, (*Z*)-β-ocimene and linalool as minor products in *in vitro* assays (Fig. 1B, Chapter 4). Transient expression of *CsTPS9* fused with a GFP reporter at the C-terminal of the protein in *Nicotiana benthamiana* leaves, suggests that the corresponding protein may be localized in the cytosol in addition to localization to the chloroplasts (Fig. 1C). *In vitro* assays using the cytosolic FPP as substrate resulted in the formation of (*E*)-β-farnesene as the most dominant sesquiterpene, but this compound is detected in trace amounts only in the induced volatile blend of cucumber (unpublished data, I.F. Kappers), which suggests that CsTPS9 *in planta* most likely acts as a monoterpene synthase. *CsLOX* was found to be upregulated by spider-mite feeding after one day of infestation as analysed by RNA-seq (Fig. 1A, Chapter 2). It represents one of the ten strongest induced genes within one day of infestation.

Cis-acting regulatory elements in promoter sequences

Putative *cis*-acting regulatory elements (CAREs) in the 2000 bp upstream sequence of the start codon of *CSTPS9*, *CsTPS19* and *CsLOX*, defined to represent the promoter (P_{CsTPS9}, $P_{CsTPS19}$ and P_{CsLOX}), were analysed using PlantCARE [*Cis*-Acting Regulatory Element (Lescot et al., 2002)]. The number of binding sites located in the 2000 bp sequences of P_{CsTPS9} was 67, 56 for $P_{CsTPS19}$ and 69 for P_{CsLOX} (Table 1). Twelve motifs were found in all three promoters, including the AAGAA, ARE, Box I, circadian, GA, G-box, HSE, MBS, Skn-1, TC-rich repeats and two non-specified motifs. Interestingly, about one third (28 – 43 %) of the motifs in all three promoter sequences were assigned to be responsive to light. The proportion of motifs associated to defence was similar (16, 18, 17%) in all three promoters. Furthermore, motifs were identified that are associated to induction by either JA or SA.

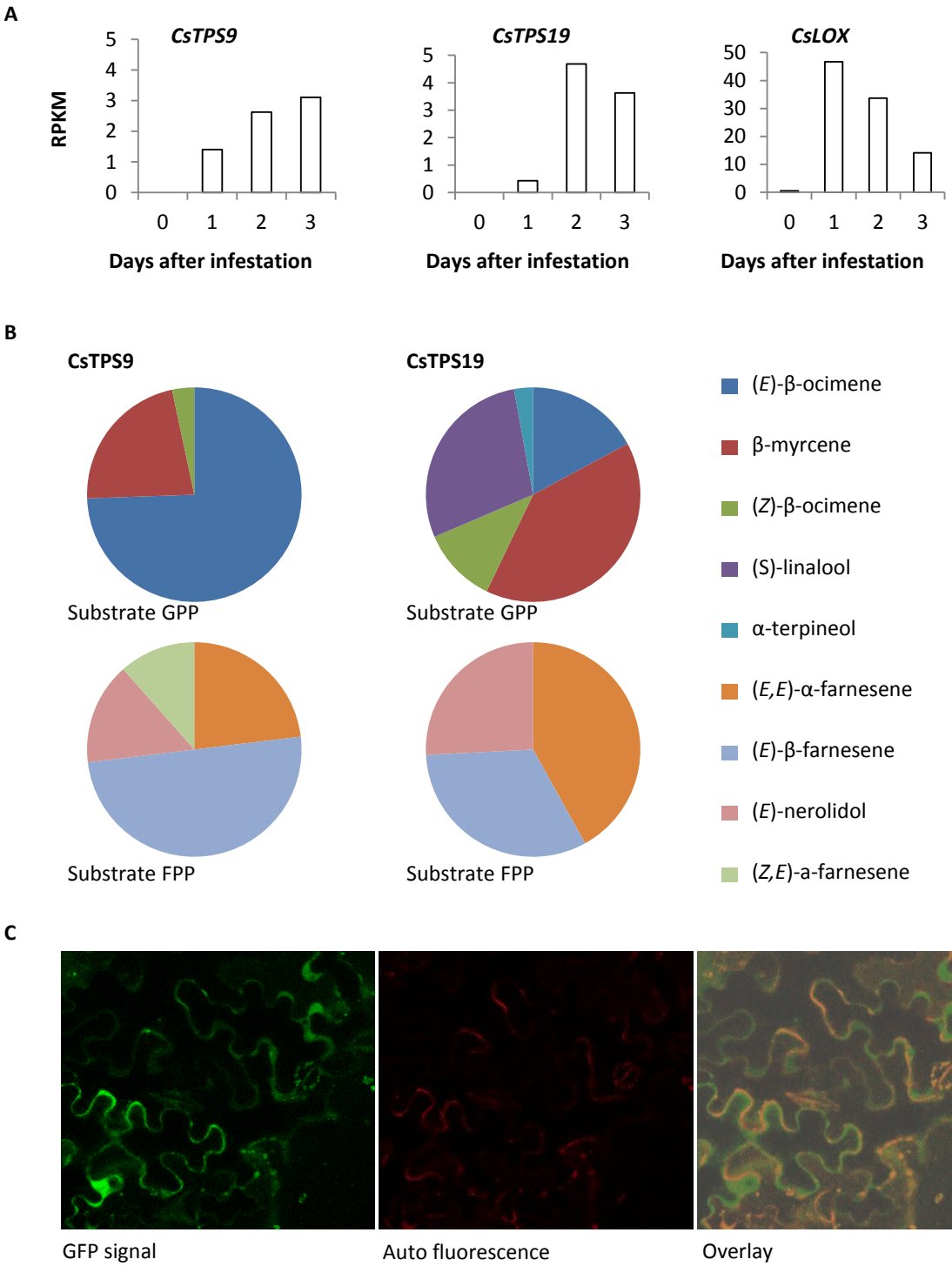

Fig. 1. Induction of *CsTPS9, CsTPS19* and *CsLOX* by spider mites and the enzymatic function of *CsTPS9* and *CsTPS19*. (**A**) Transcript abundance (RPKM) of *CsTPS9, CsTPS19* and *CsLOX* genes in leaves after infestation with spider mites for one, two or three days. (**B**) Terpene products synthesized by CsTPS9 and CsTPS19 from different precursors in *in vitro* assays. (**C**) Subcellular localization of CsTPS9::GFP fused protein in transient expression in leaves of *Nicotiana benthamiana*. Left, image of GFP; middle, image of auto-fluorescence of chlorophyll; right, overlay of GFP and auto-fluorescence.

Table 1. Percentage of motifs in the 2000 bp sequence upstream of ATG of *CsTPS9, CsTPS19* and *CsLOX17* annotated to be involved in the indicated responsiveness.

Promoter	keyword					
	light	defence	JA	SA	circadian	other
P_{CsTPS9}	28.4	16.4	7.5	9	3	41.8
$P_{CsTPS19}$	42.9	17.9	1.8	3.6	3.6	35.7
P_{CsLOX}	37.3	16.9	6.8	3.4	1.7	37.3

Differences in the level of luciferase activity between independent transgenic lines

Arabidopsis thaliana plants were transformed using *Agrobacterium tumefaciens* with a dual *β-GLUCURONIDASE (GUS)* and *LUCIFERASE (LUC)* reporter construct (Fig. S1) driven by the promoters of *CsTPS9* (*P_{CsTPS9}::GUS/LUC*), *CsTPS19* (*$P_{CsTPS19}$::GUS/LUC*) or *CsLOX* (*P_{CsLOX}::GUS/LUC*). For each of these constructs, several independent transformants were obtained except for *P_{CsLOX}::GUS/LUC* for which only one confirmed transformant line was obtained. For each of the transgenic promoter plants, the average transgene activity of LUC in cotyledons, hypocotyls and roots of T3 homozygous plants varied between independent lines (Fig. S2A). Most of the analysed transgenic lines had some *LUC* activity in the seedlings, especially in the cotyledons and the hypocotyl. An exception is line *$P_{CsTPS19}$*_D that had strong luminescence in the primary root. The luminescence of the plants decreased several hours after the application of luciferin, which may imply that pre-accumulated LUC protein was consumed. Therefore, in order to detect real-time gene expression, luciferin was pre-applied to the plants at regular intervals (during three days) in all further experiments.

Three independent transformants harbouring construct *P_{CsTPS9}::GUS/LUC* (*P_{CsTPS9}::GUS/LUC*_A,B,C) showed different levels of basal *LUC* expression in rosette stage plants (Fig. S2B) of which *P_{CsTPS9}::GUS/LUC*_B had strongest emission of photons while *P_{CsTPS9}::GUS/LUC*_A showed less luminescence and *P_{CsTPS9}::GUS/LUC*_C only emitted very few photons. Three independent lines of *$P_{CsTPS19}$::GUS/LUC* (*$P_{CsTPS19}$::GUS/LUC*_A,B,C) also differed in their basal luminescence in rosette stage plants (Fig. S3B); the luminescence of *$P_{CsTPS19}$::GUS/LUC*_A plants was ultra-high while that of *$P_{CsTPS19}$::GUS/LUC*_B and *$P_{CsTPS19}$::GUS/LUC*_C was much weaker. Moreover, *$P_{CsTPS19}$::GUS/LUC*_C showed unequal distribution of luminescence in different areas of the leaf.

Transcript levels of *CsTPS9*, *CsTPS19* and *CsLOX* all were very low in non-challenged cucumber leaves as previously analysed by RNA-seq [Fig. S3, Chapter 2, Li et al. (2011)]. Transcripts of *CsTPS9* and *CsTPS19* were low in cucumber roots and flowers under non-challenged condition. In contrast, a high level of *CsLOX* transcripts was present in root tissue and particularly in flowers. For each promoter, a transgenic line with low basal luminescence

in leaves was selected and transgenic plants harbouring *P_{CsTPS9}::GUS/LUC*, *$P_{CsTPS19}$::GUS/LUC* and *P_{CsLOX}::GUS/LUC* were analysed for expression of the reporter in different organs using GUS staining. In consensus with the transcript levels of each gene in cucumber, transgenic Arabidopsis plants of *P_{CsTPS9}::GUS/LUC*_C and *$P_{CsTPS19}$::GUS/LUC*_B showed no blue staining in roots, leaves, flowers or siliques, while *P_{CsLOX}::GUS/LUC*_C plants displayed no staining in rosette leaves, slight staining in roots and strong staining in the anthers (Fig. S3).

P_{CsTPS9}::GUS/LUC, $P_{CsTPS19}$::GUS/LUC and P_{CsLOX}::GUS/LUC are activated by infestation with spider mites

We first tested whether spider mites would feed on Arabidopsis Col-0 plants in our experimental set-up. Three-week-old plants were infested with five adult mites on the third leaf. Visual damage was observed after three days as white spots and the occurrence of dead cells was confirmed by trypan blue staining (Fig. 2A). Spider-mite damaged leaves showed blue stained spots mostly near the veins.

Plants of three independent transgenic Arabidopsis lines of *P_{CsTPS9}::GUS/LUC* (*P_{CsTPS9}::GUS/LUC*_A, B, C), one line of *$P_{CsTPS19}$::GUS/LUC* (*$P_{CsTPS19}$::GUS/LUC*_B) and one line of *P_{CsLOX}::GUS/LUC* (*P_{CsLOX}::GUS/LUC*_C) were infested with spider mites to evaluate if the LUC system is suitable to test inducible promoter activity in response to spider-mite feeding. Increased luminescence was detected after three days of infestation compared to non-infested plants of each line (Fig. 2B).

Luminescence was recorded in the third leaf of three plants of each selected line of *P_{CsTPS9}::GUS/LUC* and in *P_{35S}*::*LUC* before and three days after infestation with five spider mites placed on the third leaf. Plants that were left non-infested were also analysed at day 0 and day 3 (Fig. 3A, B). All three *P_{CsTPS9}::GUS/LUC* lines showed significant enhanced emission of luminescence in the spider-mite infested leaves compared to similar leaves of non-infested plants (Fig. 3C). Intensity of photon emission of infested leaves of *P_{CsTPS9}::GUS/LUC*_A, *P_{CsTPS9}::GUS/LUC*_B and *P_{CsTPS9}::GUS/LUC*_C was around 4-, 6- and 7-fold higher, respectively, than that of non-infested plants. No significant difference in luminescence was found between infested and non-infested *P_{35S}*::*LUC* plants (Fig. 3C).

As 35S promoter activity is high and stable, luminescence of three *P_{CsTPS9}*::*GUS/LUC* lines after mite infestation was normalized to that of *P_{35S}*::*LUC* (Fig. 3D). Luminescence of *P_{35S}*::*LUC* plants under similar conditions was higher than in any of the cucumber promoter lines, indicating that luciferin substrate and ATP availability are not limiting in the infested plants and that hence differences in luminescence reflect differences in promoter activity (Fig. S1). Transgenic Arabidopsis line *P_{CsTPS9}::GUS/LUC*_C was selected for further experiments as it was responsive to spider-mite infestation, had the lowest basal expression

and non-infested plants showed minor changes in luminescence during the experimental period.

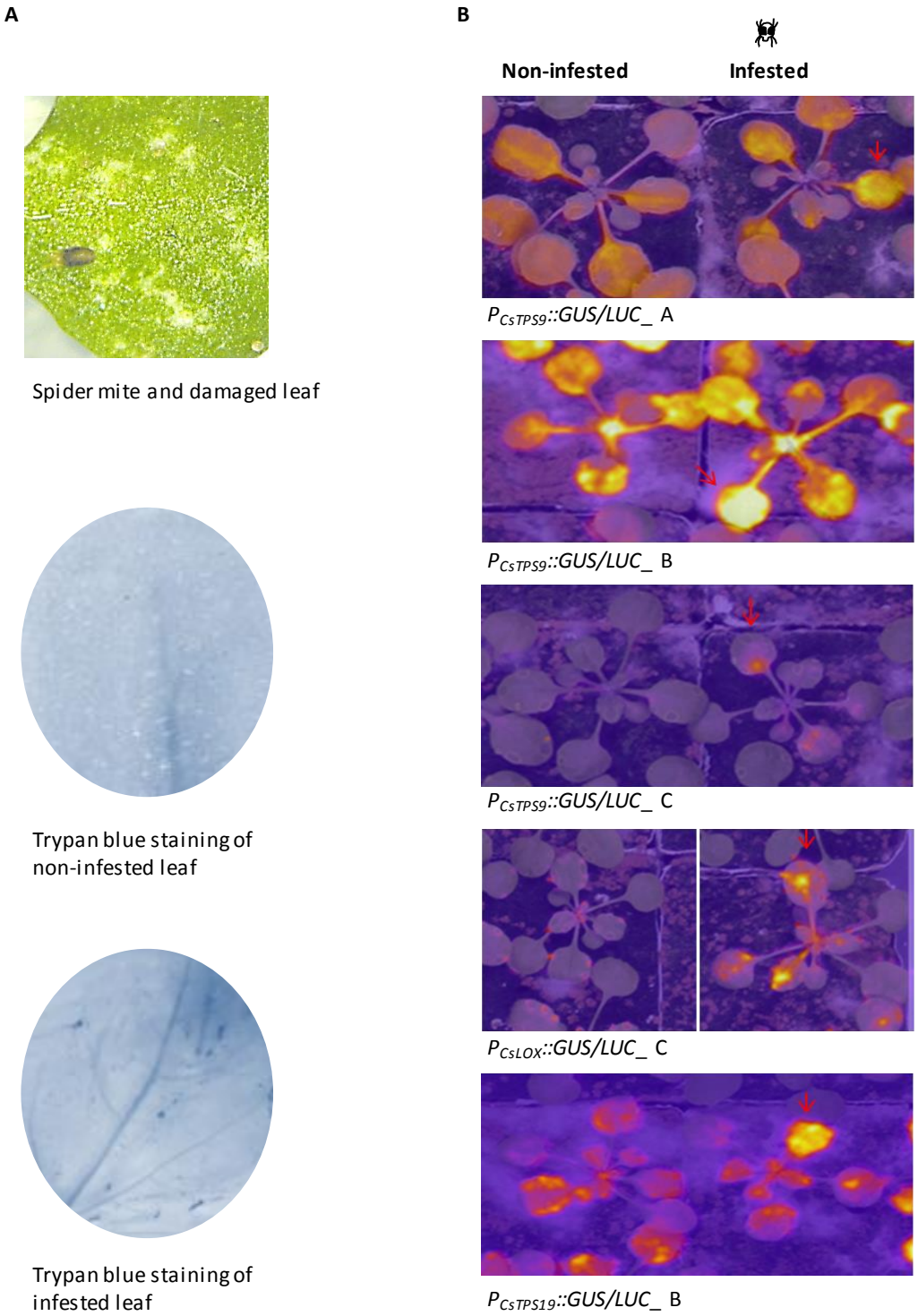

Fig. 2. Spider mites inflicted visual damage and induced luminescence in transgenic Arabidopsis lines. (**A**) Chlorotic spots as a result of feeding by spider mites in Arabidopsis Col-0. Top: A spider mite and infested area of a leaf of *Arabidopsis thaliana* Col-0 after three days of infestation; Middle: trypan blue staining of non-infested leaf; Bottom: trypan blue staining of infested leaf. (**B**) Transgenic Arabidopsis lines of P_{CsTPS9}:: *GUS/LUC*, P_{CsLOX}::*GUS/LUC* and $P_{CsTPS19}$::*GUS/LUC* showed induction of luminescence after infestation by spider mites for three days.

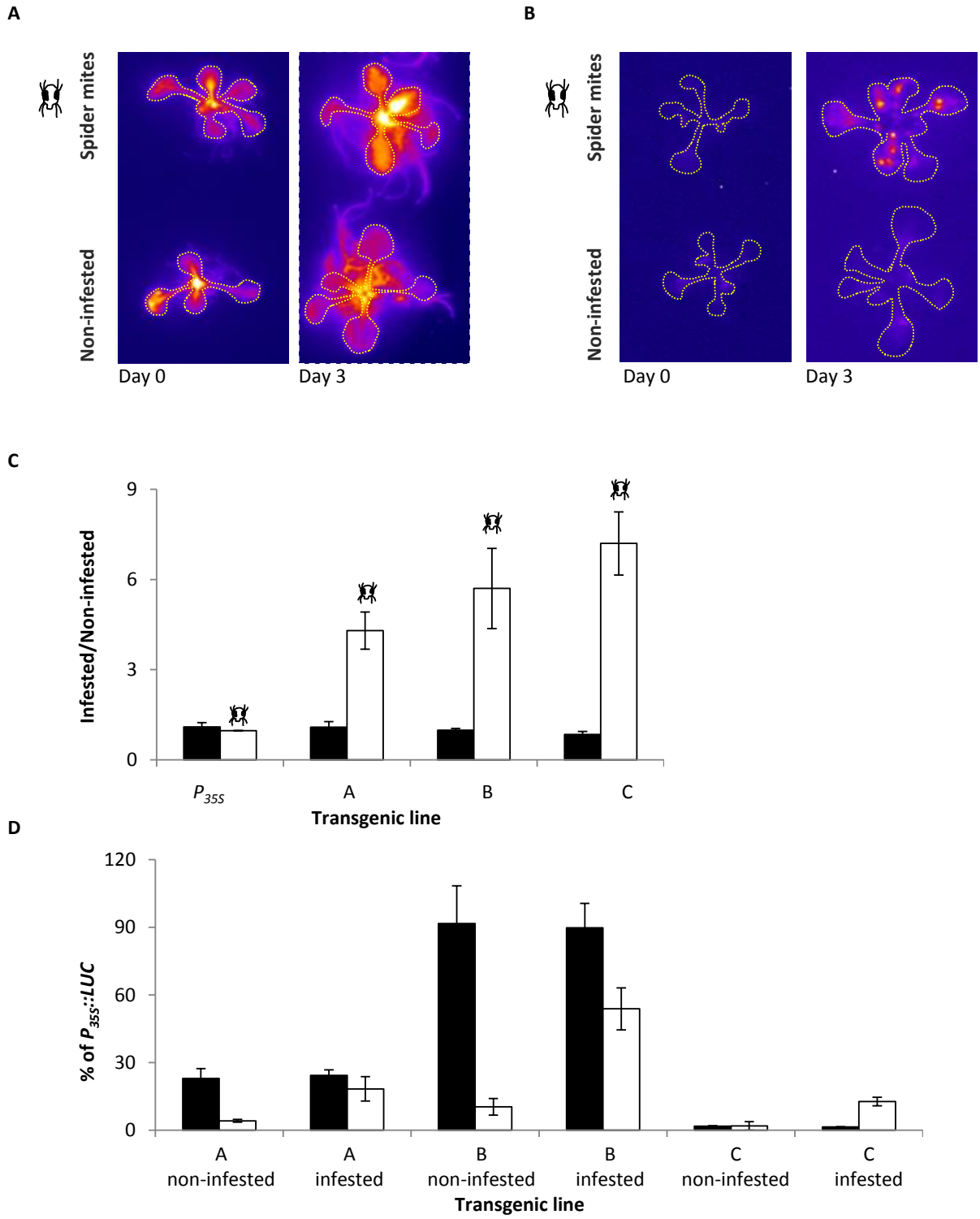

Fig. 3. Comparison of the responses of transgenic Arabidopsis plants to spider-mite infestation of three independent transgenic lines of P_{CsTPS9}*::GUS/LUC* to P_{35S}*::LUC*. (**A**) Luminescence images of a P_{35S}*::LUC* plant before and three days after spider-mite infestation. (**B**) Luminescence images of a P_{CsTPS9}*::GUS/LUC* plant before and three days after spider-mite infestation. (**C**) Luminescence intensity (means ± SD of three biological replicates) of infested plants relative to non-infested plants of P_{35S}*::LUC* (P_{35S}) and different lines of P_{CsTPS9}*::GUS/LUC* (A, B, C) before and three days after infestation. (**D**) Percentage of luminescence intensity (mean ± SD of three biological replicates) of lines of P_{CsTPS9}*::GUS/LUC* (A, B, C) compared with that of P_{35S}*::LUC* plants before and three days after infestation. In (**C**) and (**D**), black bars (■) represent the time point before infestation and white bars (□) represent the time point three days after infestation.

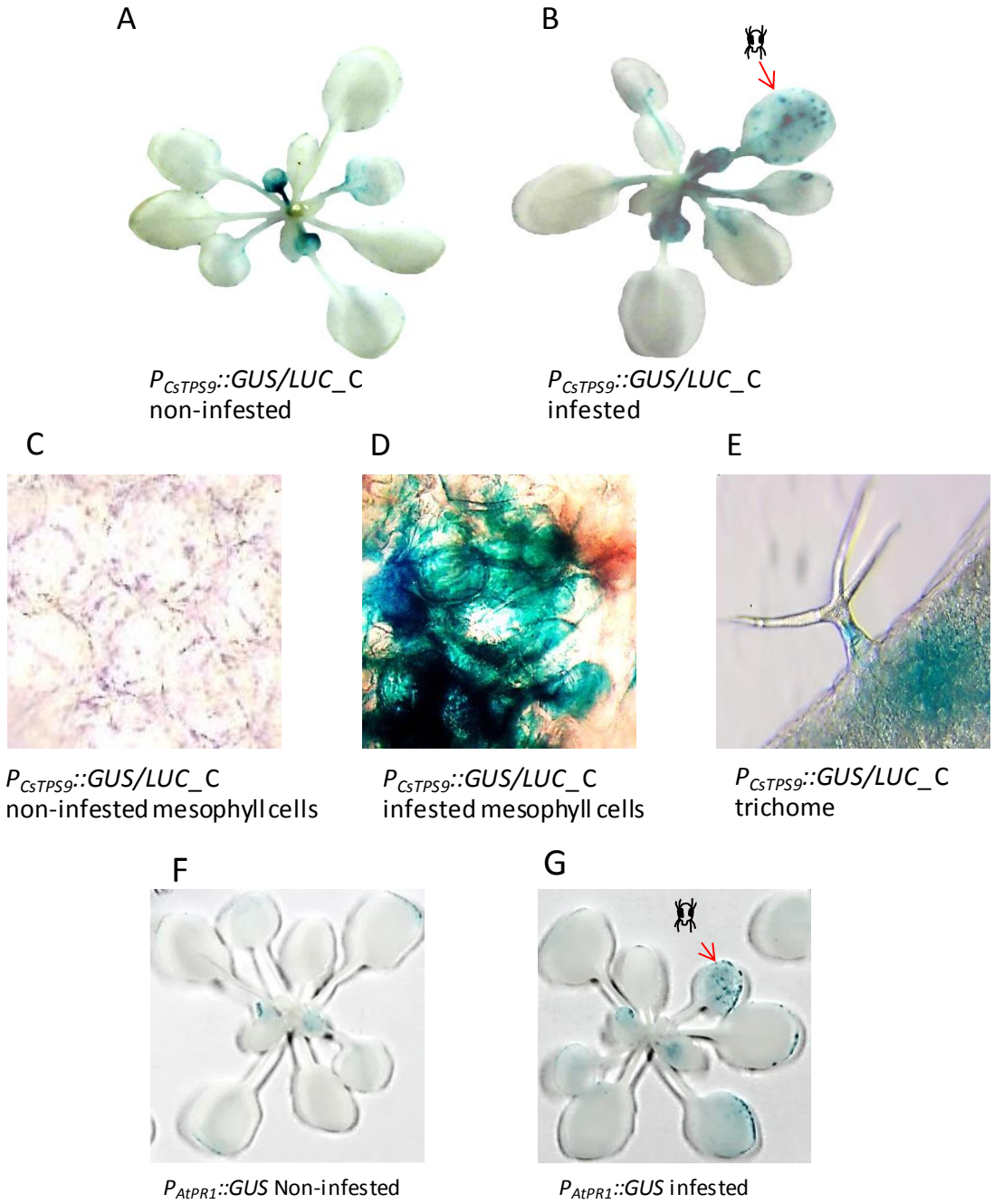

Fig. 4. GUS-staining of P_{CsTPS9}::GUS/LUC_C (**A, B, C, D, E**) and P_{AtPR1}::GUS (**F, G**) plants infested by spider mites for three days. (**A**) P_{CsTPS9}::GUS/LUC_C non-infested. (**B**) P_{CsTPS9}::GUS/LUC_C infested. (**C**) Mesophyll cells of non-infested P_{CsTPS9}::GUS/LUC_C . (**D**) Mesophyll cells of the feeding site in infested P_{CsTPS9}::GUS/LUC_C. (**E**) Stained bottom part of trichome and adjacent cells. (**F**) P_{AtPR1}::GUS non-infested plant. (**G**) P_{AtPR1} infested plant.

Histochemical GUS staining of P_{CsTPS9}::GUS/LUC_C plants showed that expression of the reporter gene was absent in non-infested plants, except for the cotyledons that in some plants stained blue (Fig. 4A). Infested plants stained blue in leaves that were damaged by spider mites in a patchy pattern corresponding to the damage spots made by the mites (Fig.

4B). Furthermore, some of the younger leaves from the same plants that were not damaged by spider mites showed staining in the petioles and sometimes in the veins. Stained cells in the infested areas were mostly located in the mesophyll layer (Fig. 4C, D). In addition, we observed that part of the trichomes located in damaged areas were stained in the basal part of the stellate trichome cells (Fig. 4E). Arabidopsis P_{PR1}::*GUS* plants were used to compare spatial and temporal expression of *PR1* (*PATHOGENESIS-RELATED PROTEIN 1*) and the promoters in this study with respect to induction by spider mites. Three days after introduction of mites on the third leaf of each plant, blue colouring was visible on the cotyledons and on the local infested leaf only (Fig. 4F, G), while P_{CsTPS9}::*GUS/LUC*_C infested plants showed blue colouring in local infested leaves but also in petioles and veins of other leaves (Fig. 4B).

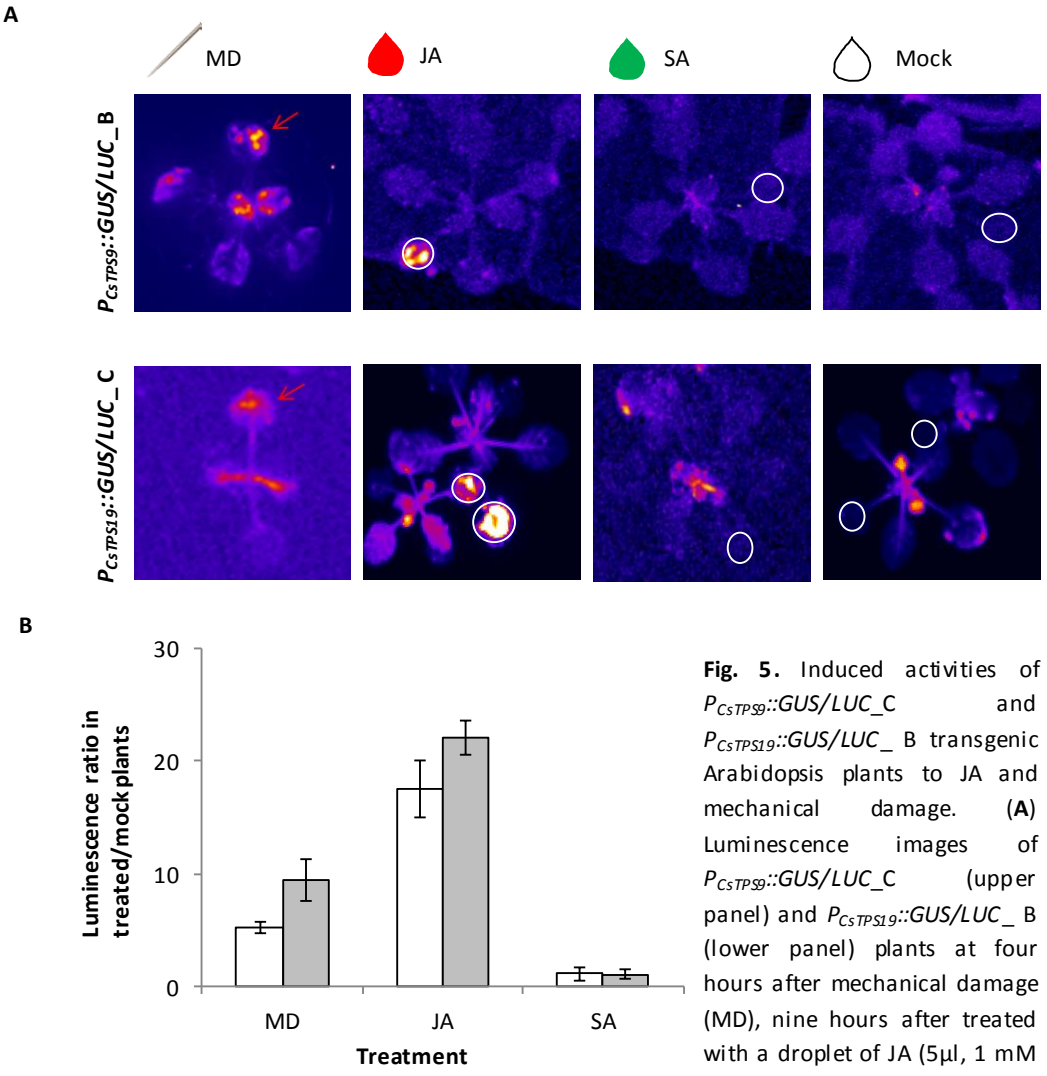

Fig. 5. Induced activities of P_{CsTPS9}::*GUS/LUC*_C and $P_{CsTPS19}$::*GUS/LUC*_ B transgenic Arabidopsis plants to JA and mechanical damage. (**A**) Luminescence images of P_{CsTPS9}::*GUS/LUC*_C (upper panel) and $P_{CsTPS19}$::*GUS/LUC*_ B (lower panel) plants at four hours after mechanical damage (MD), nine hours after treated with a droplet of JA (5µl, 1 mM with 0.01% Tween 20), with a droplet of SA (5µl, 1 mM with 0.01% Tween 20) or mock solution (0.01% Tween 20). (**B**) Luminescence intensity (mean ± SD of three biological replicates) on MD sites relative to non-MD area and luminescence intensity on sites with JA and SA relative to sites with mock solution in P_{CsTPS9}::*GUS/LUC*_C and $P_{CsTPS19}$::*GUS/LUC*_B . White bars represent P_{CsTPS9}::*GUS/LUC* and grey bars represent $P_{CsTPS19}$::*GUS/LUC*_ C.

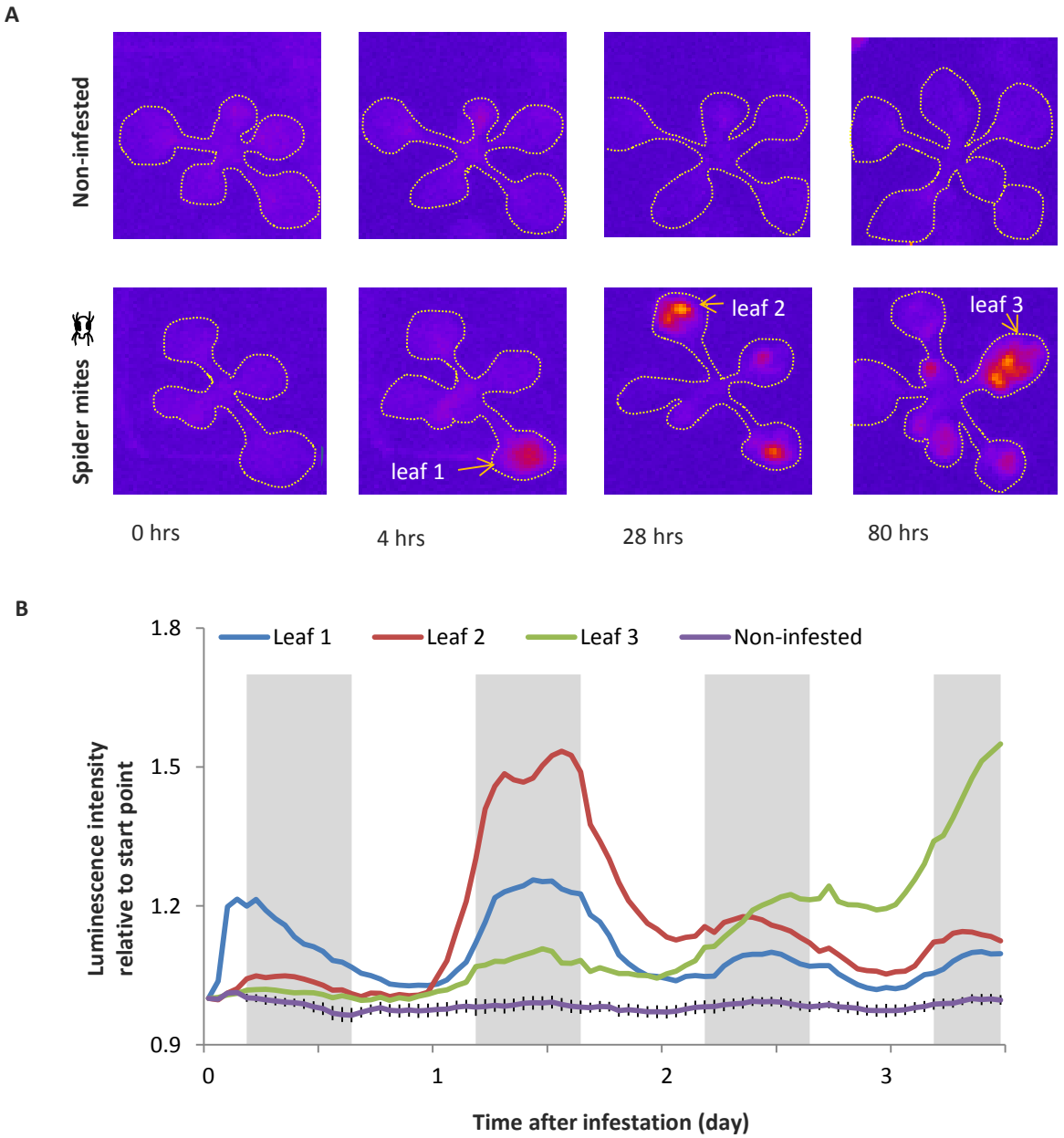

Fig. 6. Dynamic response of P_{CsTPS9}*::GUS/LUC*_C transgenic Arabidopsis plants to spider mites. (**A**) Luminescence images of P_{CsTPS9}*::GUS/LUC*_C plants infested by spider mites for different times. The first expanded true leaf of the plant was initially infested with five spider mites. (**B**) Measurement of luminescence from individual damaged leaf 1, leaf 2, leaf 3 and non-damaged leaf. Shown are data for a representative plant in multiple experiments. Grey bars indicate the dark periods. Error bars represent SD of three replicate leaves.

Dynamic response of P_{CsTPS9}*::GUS/LUC* to spider-mite feeding

P_{CsTPS9}*::GUS/LUC*_C plants were further tested for temporal dynamic responses to infestation by spider mites (Fig. 6). During the first three days of infestation, we observed that spider mites often walked from the originally infested leaf to other leaves of the same plant. In a representative experiment, five spider mites were introduced on the third leaf (cotyledons were also counted) of the plant and an increase of luminescence of the infested leaf was

observed within one hour and luminescence increased gradually during the remaining four hours of this light period (Fig. 6A, B). After the lights were turned off, luminescence decreased gradually until six hours in the subsequent second light period. During the second half of this light period, luminescence increased again reaching its maximum during the dark period at about 36 hours after the start of the experiment. This circadian rhythm was continued for the following two days but with lower amplitudes. About one day after infestation, another leaf (Fig. 6A, leaf 2) showed an increase in luminescence with a similar rhythm as the first infested leaf, but with higher amplitude during the second dark period. Light emission of this second infested leaf also decreased during the following two days. A third leaf displayed slightly enhanced luminescence during the second dark period and its amplitude increased during the third and fourth dark period. In the fourth dark period of the timespan of the experiment, leaf number 3 had the highest amplitude of luminescence compared to both other infested leaves. Visual observation after the end of luminescence monitoring showed that spider mites made visible damages on the three leaves that showed enhanced luminescence. The leaves that were visually assessed not to have been visited by spider mites and hence considered to be not directly infested, showed only basal luminescence.

Dynamic response of P_{CsTPS9}*::GUS/LUC* to infestation by aphids and thrips

The response of P_{CsTPS9}*::GUS/LUC*_ C plants to infestation by aphids (*Myzus persicae*) and thrips (*Frankliniella occidentalis*) was also analysed (Fig. 7). An increase in luminescence was observed within one hour after the introduction of thrips and maximum luminescence increase compared with the non-infested control was 73-fold, which was observed after five hours (Fig. 7B). This level of luminescence was maintained pending the 12-hour dark period and thereafter gradually decreased until 16.00, i.e. after 8 hours of light (Fig. 7B). Subsequently, photon emission increased to a second peak in the next dark period at about four hours after the lights were turned off. This peak represented the maximum level of induction during the infestation process, 83-fold higher than at the start of the experiment. After this peak, luminescence immediately decreased and did not increase again during the timespan of the experiment, although luminescence remained about 30-fold higher than in non-infested plants (Fig. 7B). During the light periods, thrips were observed for their movement on the plants and we observed that thrips moved to other leaves within two hours after the onset of infestation. After the end of recording luminescence at 55 hours since the start of the experiment, plants were observed under daylight and we saw that the originally infested leaf was seriously damaged by thrips and a number of other leaves showed silver damage spots as well (Fig. 7A). In contrast to thrips, aphids did not cause any detectable induction of luminescence on the infested plants (Fig. 7). Quantification of the dynamic luminescence emission of leaves infested by aphids did not differ from the control plants without any herbivores. Visual observation showed that aphids walked around for about two hours and then remained at the same position, which implies they were

probing/feeding (Kloth et al., 2015). As aphids feed on phloem, after 55 hours no damage was visible on the aphid-infested plants (Fig.7).

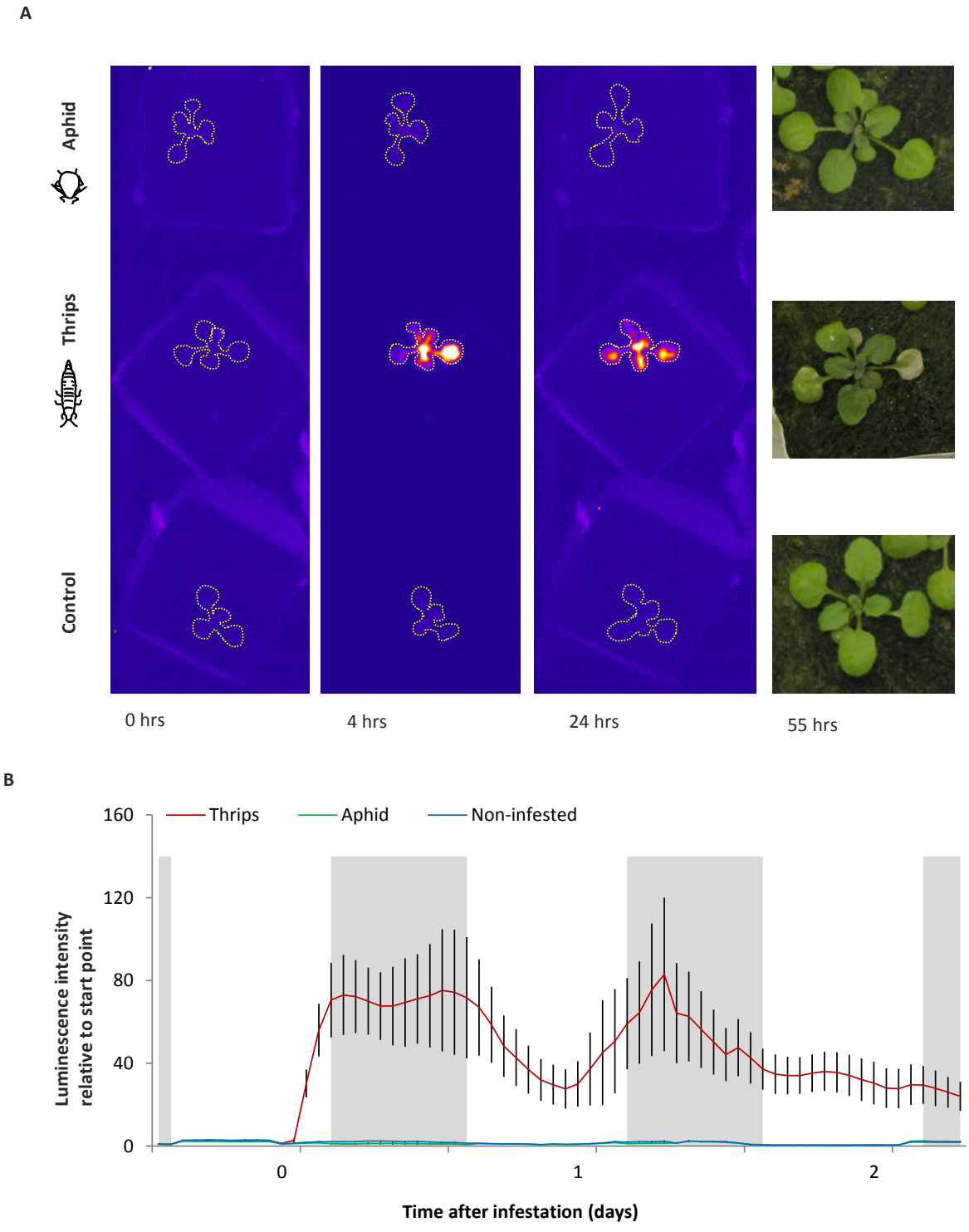

Fig. 7. Dynamic response of P_{CsTPS9}*::GUS/LUC* to infestation by aphids or thrips. (**A**) Luminescence image of P_{CsTPS9}*::GUS/LUC*_ plants infested by aphids, thrips or left non-infested for 0, 4 and 24 hours and images of the same plants under bright field at 55 hours since infestation.(**B**) Luminescence intensity (means ± SD of three biological replicates) on the plants infested by thrips, aphids or non-infested. The time when plants were infested was defined as 0 hour. Grey bars indicate the dark period.

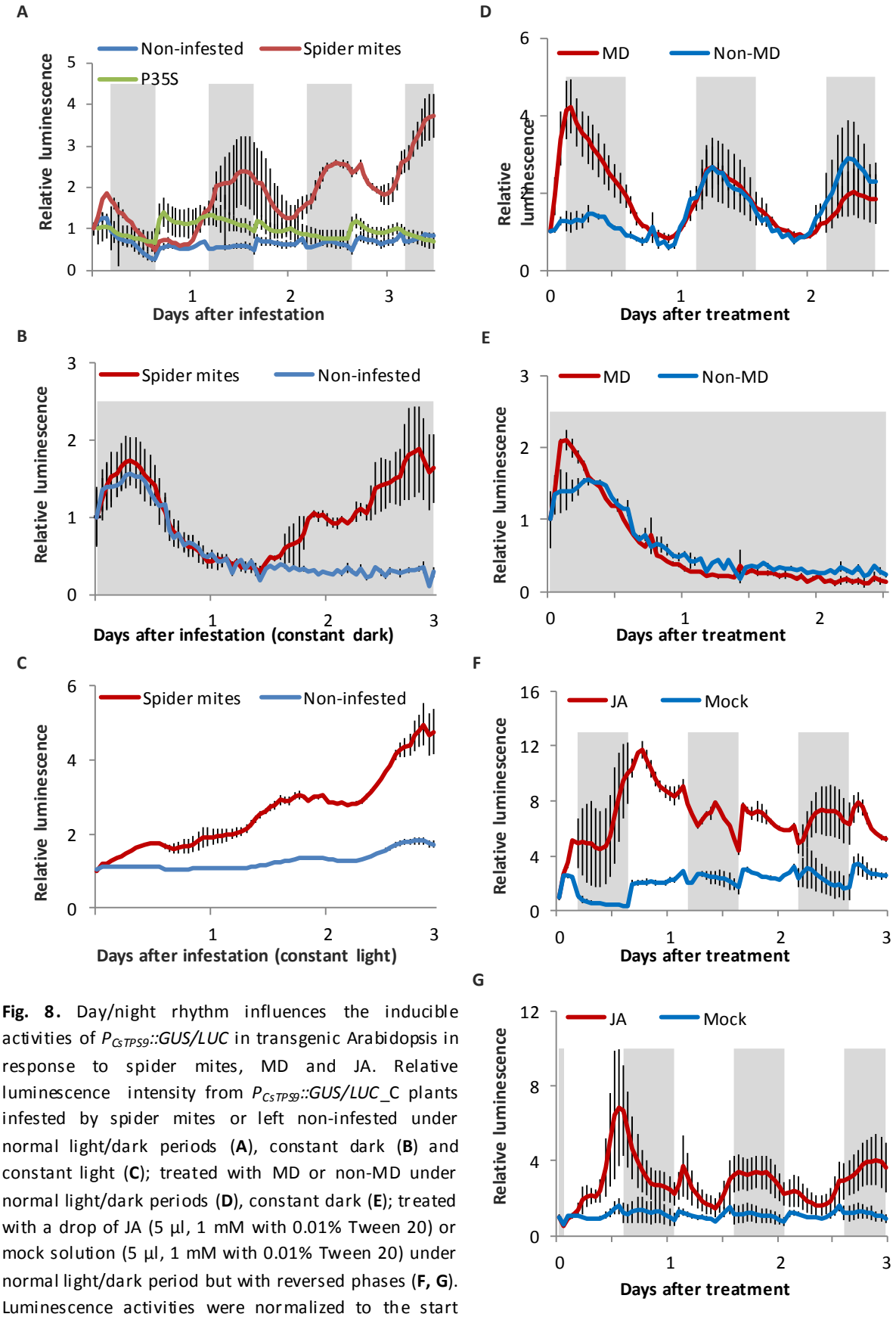

Fig. 8. Day/night rhythm influences the inducible activities of P_{CsTPS9}*::GUS/LUC* in transgenic Arabidopsis in response to spider mites, MD and JA. Relative luminescence intensity from P_{CsTPS9}*::GUS/LUC*_C plants infested by spider mites or left non-infested under normal light/dark periods (**A**), constant dark (**B**) and constant light (**C**); treated with MD or non-MD under normal light/dark periods (**D**), constant dark (**E**); treated with a drop of JA (5 µl, 1 mM with 0.01% Tween 20) or mock solution (5 µl, 1 mM with 0.01% Tween 20) under normal light/dark period but with reversed phases (**F, G**). Luminescence activities were normalized to the start point of each measured sample. Error bars represent SD of three biological replicates. Grey bars indicate the dark period.

P_{CsTPS}*::GUS/LUC* activity shows a circadian rhythm

As the temporal dynamics of LUC activity under the control of P_{CsTPS9}*::GUS/LUC* showed a nocturnal activity in response to feeding by spider mites or thrips, P_{CsTPS9}*::GUS/LUC*_C plants were analysed for changes in promoter activity during continuous light and dark conditions. We measured the total luminescence of plants during infestation to exclude variation between leaves caused by spider-mite movement within the plant. P_{CsTPS9}*::GUS/LUC*_C plants infested by spider mites under 12-h light/12-h dark cycles showed a similar circadian rhythm as described above for the individual leaves with increasing maximum luminescence during the period of infestation. Luminescence decreased during the first half of the light period followed by an increase during the second half of the light period. The increase continued during the first half of the dark period and subsequently decreased during the second half of the dark period. Within the same experiment, luminescence of transgenic P_{35S}*::LUC* Arabidopsis plants did not show any rhythm during the light and dark cycles. Next, we investigated the changes in luminescence as a result of promoter activation by spider mites in P_{CsTPS9}*::GUS/LUC*_C plants that were either infested by spider mites or were left untreated under continuous dark or continuous light conditions for three days (Fig. 8B, C). During the first six hours in constant dark conditions, both infested and non-infested plants showed an increase in luminescence. Thereafter, luminescence of both infested and non-infested plants decreased until barely detectable levels at about 34 hours in the dark. From that time onwards, luminescence of non-infested plants remained at a stable level while that of infested plants increased with circadian fluctuation during the rest of the dark period with 21 hours between maximum amplitudes at days 2 and 3. Plants became chlorotic after three days of continuous darkness. When P_{CsTPS9}*::GUS/LUC*_C plants were placed under continuous light for three days, spider-mite infested plants exhibited an increase in luminescence in a circadian rhythm and peaked during the subjective night with 30 and 26 hours between the maximum amplitudes on successive days. Under continuous light, luminescence of spider-mite infested P_{CsTPS9}*::GUS/LUC*_C plants was not restricted to the feeding spots but emitted from the whole plant and non-infested plants were induced by the constant light as well and also a vague oscillation was observed in these plants (Fig. 8B).

Mechanical damage inflicted by multiple penetrations with a needle resulted in an increase in luminescence of P_{CsTPS9}*::GUS/LUC*_C plants within the first hour and maximum levels were observed at the end of the first light period (Fig. 8D). Under a 12-h light/12-h dark cycle, a rhythmic pattern was observed with maximum luminescence at the beginning of the dark period that faded away during the two successive natural light periods. Mechanically damaged P_{CsTPS9}*::GUS/LUC*_C plants that were placed under continuous dark conditions displayed a single peak in luminescence which decreased to the level of non-damaged plants in the following days.

Dynamic luminescence in P_{CsTPS9}*::GUS/LUC*_C plants treated with JA, SA or mock solution was analysed under two contrasting light/dark cycles. First, transgenic plants were treated with

JA in the light period and luminescence emitted from the site treated with JA increased within two hours in the light. When the dark period started, the luminescence level was maintained for about six hours and then increased again with maximum luminescence about 18 hours post treatment in the light period. After that, luminescence followed a rhythm as was observed in response to infestation by spider mites or thrips. Secondly, JA was applied during the night period. Although in the first day after treatment luminescence triggered by JA showed a maximum increase during the light period which was different from the effect of herbivory induction, during the second and third day after JA-treatment a rhythm consistent with that of the herbivore infestation and the JA-treatment during the light period appeared, i.e. it peaked halfway the dark period and was lowest after about 8 hours of light.

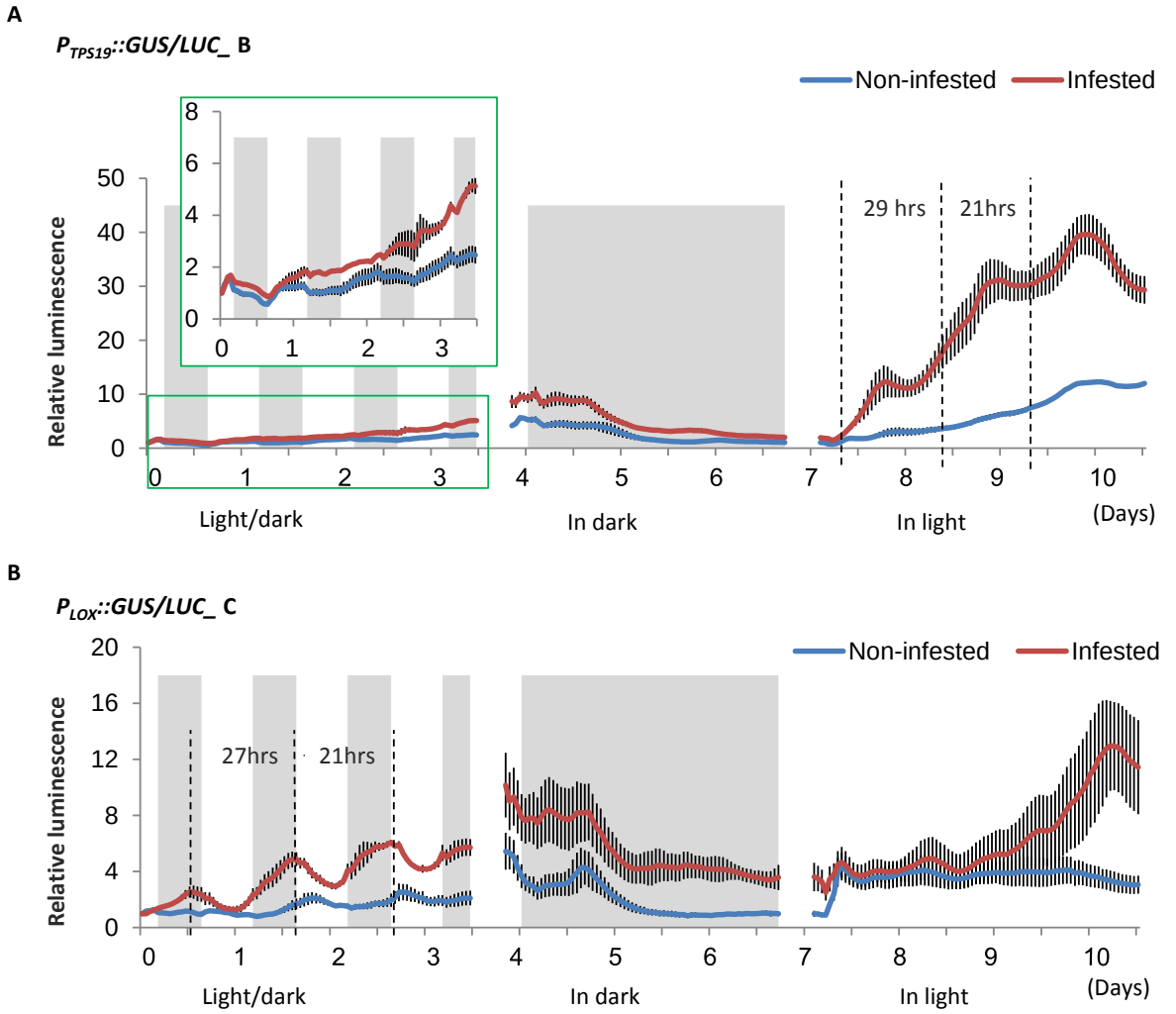

Fig. 9. Day/night rhythm influences the inducible luminescence activities of $P_{CsTPS19}$::*GUS/LUC*_B (**A**) and P_{CsLOX}::*GUS/LUC_C* (**B**) in transgenic Arabidopsis in response to infestation by spider mite. The plant infested or non-infested by spider mites were imaged for luminescence in different setup of light/dark periods. Grey bars indicate the dark period. In (**A**), the first three days are shown enlarged in the inset. Luciferin was re-applied at the beginning of each period of different light /dark pattern. Luminescence activities were normalized to the start point of each measured sample. Error bar represent SD of three biological replicates.

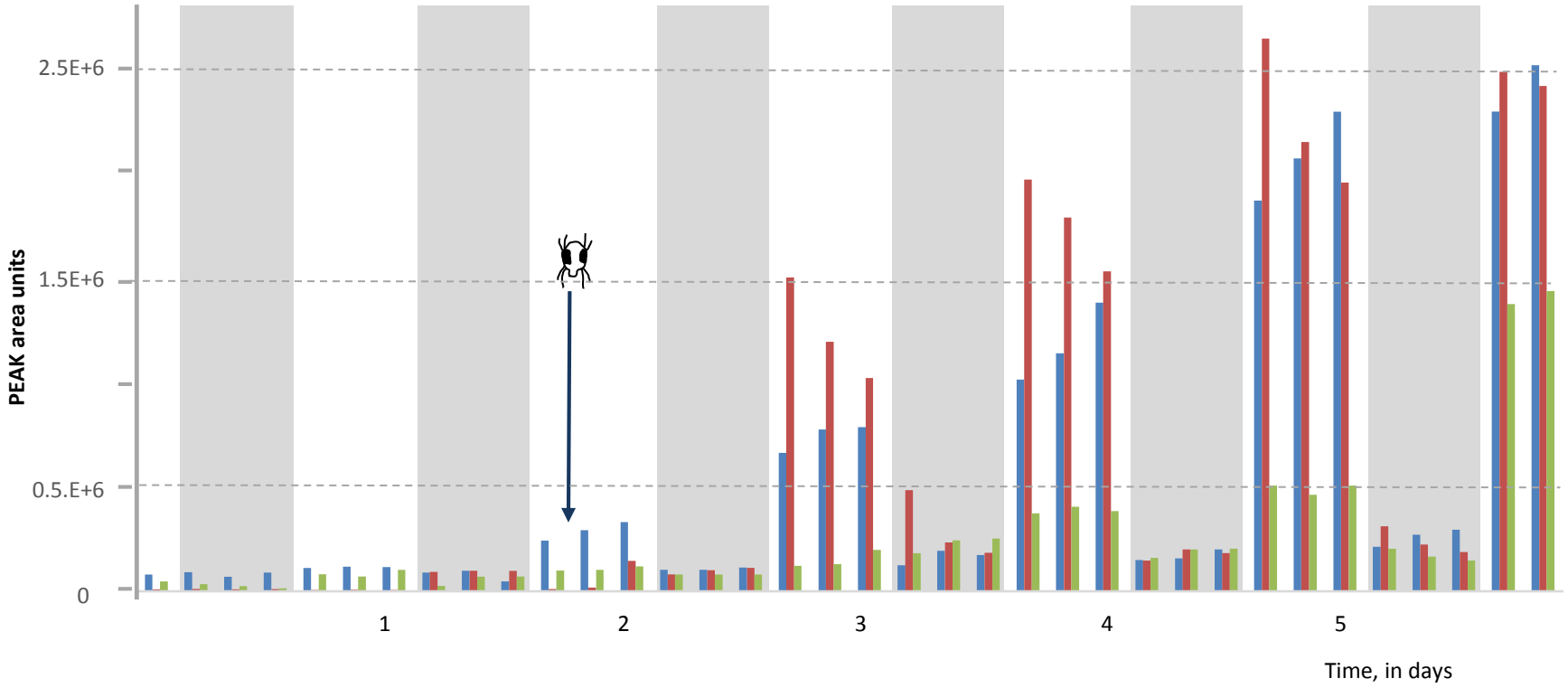

Fig. 10. Dynamic emission of volatiles from cucumber leaves infested by spider mites. Emission of green leaf volatiles (Z)-3-hexenyl acetate and 3-hexenol (blue bars ■), monoterpene (*E*)-β-ocimene (red bars ■) and sesquiterpene (*E,E*)-α-farnesene (green bars ■) by *in vitro* grown cucumber plants that were infested with spider mites in the third light period (indicated with an arrow). Each bar represents a 4-hour collection.

The results obtained for P_{CsTPS9}*::GUS/LUC_*C suggest that *CsTPS* promoters may be regulated by the circadian clock and, therefore, $P_{CsTPS19}$*::GUS/LUC* and P_{CsLOX}*::GUS/LUC* promoter activity was further investigated under successive 12-h light/ 12-h dark conditions, continuous darkness and finally continuous light conditions. Spider-mite induced luminescence of $P_{CsTPS19}$*::GUS/LUC_*B plants increased after one day of infestation, although at relatively low levels compared to non-infested plants. Exposure to continuous darkness resulted in a decrease in luminescence to the level of non-infested plants. Under subsequent continuous light luminescence of spider-mite infested plants increased in an oscillating pattern with 29 and 21 hours between the maximum amplitudes (Fig. 9A). Luminescence in transgenic P_{CsLOX}*::GUS/LUC_C* plants that were infested with spider mites increased in an oscillating pattern from the first day onwards, with maximum amplitudes at the end of the night period and 27 and 21 hours between the maximum amplitudes during two successive days. In contrast to the promoters of the terpene synthase genes, the oscillating rhythm of P_{CsLOX}*::GUS/LUC_C* plants was not maintained during continuous dark nor subsequent continuous light conditions (Fig. 9B).

Diurnal emission of spider-mite induced terpenes and green leaf volatiles

The promoters studied in the present study drive the expression of genes encoding enzymes producing (*E*)-β-ocimene, (*E,E*)-α-farnesene and the green leaf volatiles (*Z*)-3-hexenyl acetate and 3-hexenol. The production of these compounds upon spider-mite herbivory was analysed using headspace analysis on *in vitro* grown cucumber plants at four-hour-intervals followed by gas chromatography–mass spectrometry (GC-MS). (*E*)-β-ocimene, (*E,E*)-α-farnesene, (*Z*)-3-hexenyl acetate and (*Z)*-3-hexenol are compounds emitted by cucumber foliage after mite infestation and most probably the reaction products of *CsTPS9*, *CsTPS19* and *CsLOX*, respectively.

Headspace collections began one 12-h light / 12-h dark period prior to infestation, at four hours after the start of the second light period and ended four days later (Fig. 10). Non-infested cucumber plants released little, if any, detectable (*E*)-β-ocimene, (*E,E*)-α-farnesene or green-leaf volatiles. After introduction of spider mites, (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate emission increased within four to eight hours and these compounds were emitted during the following three light/dark periods with highest emission during the last four hours of the light period.

Emission of (*E*)-β-ocimene increased more than 200-fold from the beginning of the light period following the day after the onset of infestation with the highest emission at the beginning of each light period and a decrease to 10- to 30-fold higher than baseline level during the dark periods, indicating that emission of (*E*)-β-ocimene is light dependent in cucumber plants like previously described in lima bean (Arimura et al., 2008). (*E,E*)-α-

farnesene emission increased only after progressing mite infestation, and first increase in emission was observed in the third light period after the onset of infestation. Emission of (*E,E*)-α-farnesene seemed to be light-dependent as well (Fig. 10).

Discussion

We isolated the promoters of three spider-mite inducible genes which are involved in herbivory-induced volatile biosynthesis in cucumber foliage and characterized their responsiveness to various external stimuli in transgenic *A. thaliana* via a *GUS/LUC* fused reporter system. The activity of the promoters was induced by herbivory by the mesophyll cell-content feeding herbivores *T. urticae* and *F. occidentalis*, mechanical damage and JA but not by herbivory by the phloem-sucking aphid *M. persicae* nor by SA. Furthermore, we found that the promoters displays a nocturnal activity suggesting that they are under circadian control. Intriguingly, the volatiles from cucumber leaves infested by spider mites showed diurnal emission.

GUS and LUC reporter system for expression analysis of herbivores inducible genes

In this study, promoter activities were tested in transgenic Arabidopsis, using a fused reporter containing both GUS (β-GLUCURONIDASE) and LUC [LUCIFERASE, Koo et al. (2007)]. Arabidopsis accession Col-0 was used as the background for transformation of cucumber promoter-reporter constructs. Although this accession was reported not to be very susceptible to spider-mite infestation compared to other genetically and geographically diverse natural accessions (Zhurov et al., 2014), in our experiments Col-0 plants were damaged by spider mites, which was clear from the induction of chlorotic spots at the feeding sites of the mites and confirmed by trypan-blue staining of dead cells.

GFP, GUS and LUC are three commonly used reporter systems to visualize plant gene expression. GFP is useful to visualize subcellular localization of proteins but less suitable for observing gene activity on a whole plant level as auto-fluorescence is strong and varies between plant organs (Quaedvlieg et al., 1998). GUS staining is stable and has a high resolution to use for tissue-specific expression. However, the staining process destroys the cells and can, therefore, not be used to show dynamic transcriptional activity. Additionally, its low turnover rate makes it difficult to show temporal expression (Jefferson et al., 1987). The LUC reporter, on the other hand, is able to monitor real-time gene expression *in planta* in a sensitive and rapid way, but is not useful to detect tissue- or cell-specific activity. To combine the advantages of GUS and LUC reporters, a GUS/LUC fused reporter system was developed and shown to be functional in gene expression studies in Arabidopsis (Koo et al.,

2007). With this dual reporter system, the dynamic temporal activity of *P_{CsTPS9}::GUS/LUC*, *$P_{CsTPS19}$::GUS/LUC* and *P_{CsLOX}::GUS/LUC* in response to herbivory, hormones and wounding was observed at the whole plant level using luminescence. Furthermore, spatial promoter activity was visualized by histochemical staining which showed mesophyll- and trichome-specific expression of *P_{CsTPS9}::GUS/LUC.* GUS staining also showed the high activity of *P_{CsLOX}::GUS/LUC* in anthers. Moreover, the LUC reporter was more sensitive compared to GUS-staining as weak luminescence was detected from *P_{CsTPS9}::GUS/LUC*_C and *$P_{CsTPS19}$::GUS/LUC*_B plants while GUS-staining of these plants did not show any colouring.

Therefore, promoter activities in this study were mainly studied using the LUC reporter. Luminescence of transgenic plants is not only influenced by the activity of its controlling promoter but also by the availability of ATP and luciferin substrate which are involved in the reaction catalysed by luciferase to emit photons (Fig. S1). Non-saturating levels of ATP and luciferin in the plant cell may therefore lead to false interpretation of promoter activity. Availability of ATP could be limited during dark periods and hence absence of photosynthesis. In experiments with intact plants, luciferin is normally sprayed to the surface of the plants and absorbed into plant cells where the reaction occurs. When promoter activities are too strong and/or too high levels of luciferase proteins are present in the cell, the influx of luciferin in healthy plants may not be sufficient to saturate the proteins. In this scenario, damaging the surface of the plant by either wounding or herbivory could enhance the influx of luciferin and result in a burst of luminescence which hence does not represent the activity of the promoter (Nass and Scheel, 2001). To ensure that luminescence emitted by the transgenic plants in our study represents the activity of the introduced promoters, lines with low basal luminescence were selected and their luminescence compared to that of transgenic plants harbouring the strong constitutive CaMV35S promoter (*P_{35S}::LUC)*. Even after induction by herbivory or application of MD or phytohormones, luminescence in plants of selected transgenic lines was always lower than that of *P_{35S}::LUC* plants indicating that the activity of the tested promoters is correctly represented by the dynamics of luminescence. Another point of concern is that luciferin or one of its breakdown products may act as an analogue of SA and hence could trigger the expression of SA-responsive genes (Jorda and Vera, 2000). However, the selected lines in this study showed just detectable luminescence when only luciferin but no stresses were applied. Additionally, the promoters were also not triggered by the application of SA, suggesting that the cucumber promoters analysed in this study were not affected by the luciferin applied.

Involvement of JA but not SA in the response to cell-feeding herbivores

P_{CsTPS9}::GUS/LUC and *$P_{CsTPS19}$::GUS/LUC* Arabidopsis plants were responsive to JA but not to SA. A single treatment with JA on a localised spot of a leaf resulted in higher promoter activities than control plants during the second and third day after application. JA is induced

by spider-mite infestation in multiple species including lima bean (Dicke et al., 1999), tomato (Ament et al., 2004) and cotton (Miyazaki et al., 2014). Furthermore, methyl salicylate (MeSA) was emitted upon spider-mite herbivory by multiple species including lima bean (Dicke et al., 1990), tomato (Ament et al., 2004) and cucumber (Kappers et al., 2011) and SA was induced by mite herbivory in frijole (*Phaseolus vulgaris,* He et al., 2007)). Both JA and SA increased in Arabidopsis after infestation by spider mites (Zhurov et al., 2014). In cucumber, JA induces a blend of volatiles which is qualitatively similar to the blend induced by spider mites (Kappers et al., 2010) and the content of JA showed a burst around two hours after infestation after which it returned to base-line levels around four hours after infestation (Chapter 2). In contrast, the SA concentration decreased 2-fold around two hours after the onset of infestation (Chapter 2). Although SA application did not trigger any response of the reporters driven by the tested promoters, the promoter of the SA-responsive gene *PR1* did respond to spider-mite infestation. Indeed, SA biosynthesis and signalling genes were induced in Arabidopsis infested by spider mites but SA-related deficiency mutants did not display significantly altered resistance (Zhurov et al., 2014). Although the SA-regulation network may play a role, it appears that JA dominates the regulation of cucumber inducible defence to spider mites.

Multiple *cis*-acting regulatory elements (CAREs) present in the promoter sequences included in this study, were identified as possibly involved in JA or SA responsiveness. For example, three G-boxes, which are possibly required for JA mediated expression regulation (Kim et al., 1992, Mason et al., 1993, Endt et al., 2007) were present in P_{CsTPS9} and one was present in $P_{CsTPS19}$. Moreover, a W-box possibly associated to responsiveness to SA was present in both P_{CsTPS9} and $P_{CsTPS19}$. Whether motif(s) in these promoters really function as binding sites to potential transcription factor(s) and under which conditions, is still unclear and was not the purpose of our study. However, the presence of these motifs allows promoters to be bound by the potential transcription factors in the signalling pathways of JA or SA and hence can be regulated.

Influence of different feeding styles

Spider mites and thrips activated P_{CsTPS9}*::GUS/LUC* but aphids did not. Aphids are phloem feeders. Thrips belong to the same feeding guild as spider mites, i.e. they feed on the content of mesophyll cells using their stylets, resulting in silver-white spots on infested leaves. Spider-mite infestation results in chlorotic spots while aphids do not inflict visible damage. Herbivores with different feeding habits can induce different transcriptional responses. Comparison of the up- and down-regulated genes in Arabidopsis infested by the chewing generalist *Plutella xylostella,* chewing specialist *Pieris rapae*, chewing generalist *Spodoptera litoralis*, cell sucking generalist *F. occidentalis*, phloem feeding generalists *Bemisia tabaci* and *M. persicae*, and phloem feeding specialist *Brevicoryne brassicae* showed

that the most similar transcriptional responses were induced by *P. xylostella* and *S. littoralis*, which both are leaf-chewing caterpillars, that consume relatively large amounts of tissue (Ehlting et al., 2008, De Vos et al., 2005, Reymond et al., 2004, Kempema et al., 2007, Little et al., 2007, Kusnierczyk et al., 2007), while the cell-feeding *F. occidentalis* and the phloem-feeding *B. tabaci* and *M. persicae*, which inflict smaller or even invisible physical damage to the plant tissue, caused more different transcriptional changes compared to *P. xylostella* (Ehlting et al., 2008, De Vos et al., 2005, Reymond et al., 2004, Kempema et al., 2007, Little et al., 2007, Kusnierczyk et al., 2007). In our study, we selected promoters of cucumber genes that were strongly and early induced by spider mites. Transgenic Arabidopsis plants expressing the P_{CsTPS9}*::GUS/LUC* reporter construct responded to infestation by spider mites and thrips, but not to phloem-feeding aphids, indicating that the promoter of *CsTPS9* responds to cell-content feeding herbivores. Considering that P_{CsTPS9}*::GUS/LUC* was also activated by mechanical damage, a shared up-regulation in the response to spider mites and thrips could be due to the fact that both herbivores cause mechanical wounding. Indeed, thrips inflicted more damage than spider mites and correspondingly thrips induced stronger luminescence in P_{CsTPS9}*::GUS/LUC* plants. Thrips upregulated the luminescence in P_{CsTPS9}*::GUS/LUC* plant 83-fold, while the maximum induction by spider mites and MD was only about 4-fold and by JA about 13-fold in the same time span. Limited, one-time, mechanical damage quickly activated the promoter, which then decreased to the control level within one day. Repetitive mechanical damage of lima bean using a robot (MecWorm) resulted in an induced volatile blend that was strikingly similar in quality to the blend induced by the feeding of real herbivores (Mithofer et al., 2005), suggesting that repetitive mechanical damage that is inflicted during herbivory is sufficient to trigger the biosynthesis of herbivory inducible volatiles in plants.

Local and systemic defence

Luminescence of transgenic P_{CsTPS9}*::GUS/LUC*, $P_{CsTPS19}$*::GUS/LUC* and P_{CsLOX}*::GUS/LUC* plants was induced at the locally infested sites but not detected in systemic leaves or other plant organs. Plant defence can be induced both locally and systemically. Systemic responses upon herbivory were found in e.g. corn (Turlings and Tumlinson, 1992) and cotton (Rose et al., 1996). In both these studies, emission of volatiles increased in leaves which were distal to the feeding site, although the blend of volatiles emitted by systemic leaves contained fewer terpenoids compared to that emitted by the infested leaves. Feeding by spider mites on the cotyledons of cucumber plants resulted in an increase in the concentration of the defensive metabolite cucurbitacin C in the first systemic leaf (Agrawal et al., 1999). However, in our study the induction of the promoters was localized to the infested sites. Although the areas with high luminescence shifted to different places on the infested leaves or from the original infested leaf to neighbouring leaves, this is most likely caused by the fact that the herbivores migrated from the original site to other places. Moreover, induced luminescence of the

transgenic plants remained strictly localized to the sites that were treated with JA or mechanically damaged. Confusingly, GUS reporter expression of infested *P_{CsTPS9}::GUS/LUC*_C plants was sometimes enhanced in the petioles of undamaged leaves indicating differential regulation at the local and systemic level. In the same experiment, *P_{PR1}::GUS* plants showed only detectable staining in response to SA on local infested leaves. *PR1* is regulated by SA and a reporter of systemic defence against pathogens (Cameron et al., 1999). Considering JA but not SA-triggered activity of *P_{CsTPS9}::GUS/LUC* in our study, current findings will have to be complemented with more in-depth studies to elucidate whether promoters of herbivore-inducible *TPS* genes act systemically.

Regulation of *P_{CsTPS9}::GUS/LUC*, *$P_{CsTPS19}$::GUS/LUC* and *P_{CsLOX}::GUS/LUC* by light and the circadian clock

The activity of the promoters of the three defence-related genes displayed rhythmic oscillation during the light and dark periods. To ensure the rhythms correctly represent the promoter activities, *P_{35S}::LUC* transgenic Arabidopsis plants were analysed under the same conditions. Although luminescence emitted from *P_{35S}::LUC* plants showed small changes over the light and dark periods, the pattern was clearly different from *P_{CsTPS9}::GUS/LUC*, *$P_{CsTPS19}$::GUS/LUC* and *P_{CsLOX}::GUS/LUC* plants. Considering that the expression of P_{35S}*::LUC* should be highly stable, the small changes in luminescence that we see may indicate changes in the energy level as during the light periods the plants cells may have higher levels of ATP from photosynthesis, essential for the reaction catalysed by luciferase to emit photons (de Ruijter et al., 2003). Differences in luminescence from *P_{CsTPS9}::GUS/LUC*, *$P_{CsTPS19}$::GUS/LUC* and *P_{CsLOX}::GUS/LUC* plants that were infested with spider mites or thrips or were treated with JA had a much wider range of fluctuation than observed for the *P_{35S}* plants. This confirms that the rhythms observed for the transgenic plants we studied here were not a consequence of changes in the energy level or access to the luciferase substrate, which are two important factors that may influence luminescence from plants. Another possible factor which may influence the luminescence rhythms shown by plants infested by spider mites or thrips is the behaviour of the herbivores. Herbivores often display rhythmic feeding. For example, the feeding of cabbage looper (*Trichoplusia ni*) caterpillars is circadian-regulated and it feeds mostly during the day (Goodspeed et al., 2012). There is little known about spider-mite and thrips feeding during day and night. However, application of JA involves no herbivore behaviour but the luminescence from transgenic plants still showed a nocturnal activity. Obviously, therefore, regardless of whether feeding behaviour of spider mites and thrips may or may not have an influence on the rhythms we observed, the activities of the tested promoters were essential for the rhythms in the assays.

Rhythmic emission of volatiles and expression of genes involved in their biosynthesis have been reported in multiple species. MeJA induced emission of terpenes including oxygenated

monoterpenes, monoterpene hydrocarbons and sesquiterpenes, as well as methyl salicylate from Norway spruce followed a diurnal activity with higher release during the light period in a time course over seven days (Martin et al., 2003). Lima bean leaves that were mechanically damaged during the day resulted in maximal emission of β-ocimene and (*Z*)-3-hexenyl acetate in the late photophase while nocturnally applied mechanical damage only triggered nocturnal emission of (*Z*)-3-hexenyl acetate but low amounts of β-ocimene which then burst after onset of the light phase (Arimura et al., 2008). Three-hourly collection and analysis of volatiles from healthy and leafminer (*L. huidobrensis*) infested kidney bean (*Phaseolus vulgaris)* plants showed that undamaged plants released trace amounts of volatiles with no obvious rhythm while infested plants released higher levels of volatiles with a clear rhythm which peaked at the end of the day (Sufang et al., 2013). The expression of *A. annua QH6,* encoding an (-)-α-PINENE/(-)-β-PINENE synthase, is diurnally regulated, although it was transiently reduced by mechanical wounding or application of a fungal elicitor (Lu et al., 2002). In contrast to diurnal emission of defence-related volatiles emitted from foliage with highest emissions during light periods described in the above examples, emission of scent volatiles including major components such as linalool, (*E*)-β-ocimene and other monoterpenes from flowers of *Lilium auratum* followed a nocturnal emission pattern with low release rates during the light period and high release rates during the dark period (Kong et al., 2012). In our study, we tested the promoters of *CsTPS9* and *CsTPS19,* two terpene synthase genes which are inducible in cucumber by infestation of spider mites. *CsTPS9* encodes a terpene synthase that can convert GPP to (*E*)-β-ocimene, β-myrcene and (*Z*)-β-ocimene and FPP to (*E*)-β-farnesene, (*E,E*)-α-farnesene, (*E*)-nerolidol, and (*Z,E*)-α-farnesene while *CsTPS19* encodes a terpene synthase able to produce similar terpenes but with different ratio. CsTPS9 is more specifically making (*E*)-β-ocimene and CsTPS19 predominantly makes (*E,E*)-α-farnesene, two major terpenoid components within the spider-mite induced blend of cucumber. Both terpenoids as well as (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol showed a diurnal emission after induction by spider mites. Next to circadian rhythmicity, promoter activation was shown to be light dependent as well, as circadian rhythmicity eventually disappeared in complete darkness, while under continuous light the amplitudes of the luminescence increased. However, in continuous darkness the energy level in plant leaves is expected to decrease and have general negative consequences for the transcription of plant genes as well as the reaction catalysed by luciferase to emit photons. Moreover, both continuous darkness and light are stresses to plants and therefore could influence the promoter activities of the defence-related genes.

Indeed, multiple light-associated CAREs are present in the promoters we tested. The light responsive element box I (Yamada et al., 1994) was present in all three promoters, at multiple sites. The G-box present in each of the promoter sequences could be essential for light regulation as well (Martinez-Hernandez et al., 2002, Lopez-Ochoa et al., 2007). A motif called “circadian” [CAANNNNATC, *cis*-acting regulatory element involved in circadian control, Piechulla et al. (1998)] was present at -847 bases and -587 bases of the promoter sequence

of *CsTPS9*, at -1054 bases and -347 bases of that of *CsTPS19*, and at -301 bases of that of *CsLOX*.

Our results suggest that the promoters tested induce gene expression such that it peaks in the night, while in contrast, the emission of the corresponding terpenes and green leaf volatiles occurs mainly during the light period. Possibly, high expression of the *CsTPS*9, *CsTPS19* and *CsLOX* during the night results in the accumulation of active enzymes which are 'ready-to-go', when enough substrate becomes available at the onset of the day and subsequently results in volatile emission for the production of energy-costly secondary metabolites, only during the day. This is in agreement with the burst of emission of β-ocimene upon onset of light, by lima bean plants which were previously damaged in the dark period (Arimura et al., 2008). In lima bean plants, the expression of the gene encoding β-OCIMENE SYNTHASE (*PlOS*) was regulated by damage via JA accumulation at wounded sites and the biosynthesis of β-ocimene was dependent on the fixation of CO_2 by the photosynthesis in the plastid (Arimura et al., 2008), where the MEP/DOXP pathway which synthesizes the terpenoids precursors GPP and GGPP is also located. The expression of the genes of the MEP/DOXP-pathway is dependent on light in all investigated plant species (Hemmerlin et al., 2012). Indeed, expression of almost all MEP-pathways genes in Arabidopsis seedlings was repressed in darkness (Hsieh and Goodman, 2005). The expression of MEP-pathways genes encoding a DXP-SYNTHASE (1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE) and two GPP SYNTHASES was upregulated in cucumber leaves by spider-mite infestation (Chapter 2). In conclusion, it is not unlikely that the supply of precursors determined the diurnal emission of terpene volatiles in cucumber leaves triggered by spider-mite herbivory. To verify this, the rhythmicity of the expression of the genes encoding the precursor supply pathways should be evaluated.

Material and Methods

Herbivores

Two-spotted spider mites (*Tetranychus urticae*) were provided by Koppert BV (The Netherlands) and reared on lima bean (*Phaseolus lunatus*) for many generations. For experiments female adult mites were selected using a stereo microscope. Aphids (*Myzus persicae*) and thrips (*Frankliniella occidentalis*) were obtained from the Laboratory of Entomology, Wageningen University. Aphids were reared on radish (*Raphanus sativus*) and wingless adults were used. Thrips were reared on green pods of broad bean (*Vicia faba*) and five-day-old larvae were used for experiments. Herbivores were transferred to the plants using a fine brush with soft hairs and we visually checked after 10 minutes if herbivores were alive.

Putative *cis*-element analysis

To analyse sequences of selected promoters (P_{CsTPS9}, $P_{CsTPS19}$ and P_{CsLOX}), their 2000 bp upstream sequences from the initiation start were analysed for the presence of *cis*-acting elements using an online service PlantCARE [Cis-Acting Regulatory Element, Lescot et al. (2002)]. Aligned motifs for each promoter were listed as their distances to the start codon of the gene.

Construction of promoter::reporter constructs and transgenic *A. thaliana* plants

Sequence information of the promoters used in this study was obtained from the reported cucumber genome [Version 2, (Huang et al., 2009, Li et al., 2011)]. The promoter regions of *CsTPS9* (*Csa2M299880*), *CsTPS19* (*Csa3M095040*) and *CsLOX* (*Csa2M024440*), respectively, were directly synthesized into vector pMK-RQ (Thermo Fisher Scientific Inc, USA) using the genomics sequence of *Cucumis sativus* 9930 (Chinese Long) as a template. P_{CsTPS9}*::GUS/LUC*, $P_{CsTPS19}$*::GUS/LUC* and P_{CsLOX}*::GUS/LUC* cover the genomic region from -2000 to 0; -1000 to 0 and -1974 to 0 nucleotides from the translation start point, respectively. Plasmids were transformed into *E.coli* strain DH5α and multiplied. Subsequently, plasmids were extracted and digested using restriction enzyme Pst I at the 5' end and BamH I at the 3' end for each fragments and assembled into a binary vector pGUS/Luc3300 in front of the GUS/LUC fusion reporter gene using T4 DNA ligase (Invitrogen, USA). Plasmids were transformed into *E.coli* strain DH5α, multiplied, extracted and sequenced to get the right clones. Plasmids with correct sequences were transformed into *Agrobacterium tumefaciens* strain Agl0. The transformed Agrobacterium strains were used for stable transformation of *Arabidopsis thaliana* Col-0 via floral dipping (Logemann et al., 2006). Transgenic plants were selected *in vitro* on half Murashige and Skoog [MS, Murashige and Skoog (1962)] agar plates containing 50 mg/L of BASTA (glufosinate ammonium). T1, T2 and T3 transgenic plants were selected using 50 mg/L of BASTA. Homozygous plants were identified by selecting T3 plants from T2 lines that did not segregate in resistance to BASTA on MS.

For subcellular localization of *CsTPS9*, the coding sequence without the stop codon (TAA) of the gene was amplified using polymerase chain reaction (PCR) using forward primer: 5'-ATGGCTATTCTTCATCATCCTCTTC-3' and reverse primer: 5'-AGCAGGCTTGATAAATAAGGATAA-3'. The template for the reaction was a cDNA library made from cucumber leaves that were induced by JA. The amplified fragment was cloned into an entry TOPO® Vector using TOPOPcr^{tm}8/GW/ TOPO® TA cloning kit following the manual (Invitrogen, USA) and transferred into *E.coli* strain DH5α. Plasmids were isolated, sequenced and constructs with correct insertions were selected. Fragments were cloned into a Gateway binary vector pK7FWG2.0 in front of an Enhanced Green Fluorescent Protein (*EGFP*) gene lacking the start codon following the manual (Invitrogen, USA). The construct was transferred into

Agrobacterium tumefaciens strain Agl0. For GFP assay, leaves of five week old *Nicotiana benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* strain Agl0 in buffer (MgCl2: 500mM; MES-KOH: 500mM; Acetosyringone: 100mM) using a 1ml syringe. Three days after infiltration, leaves were cut off and observed under a laser scanning confocal microscope (Leica SP2). GFP was excited at 488 nm and detected at 505-530 nm, while for auto fluorescence excitation was at 488 nm and detection at 570-630 nm.

Treatment of wounding, herbivory and phytohormones

For the comparison of three independent transgenic lines of P_{CsTPS9}*::GUS/LUC_* to P_{35S} in their responses to spider-mite infestation, seeds were surface sterilised, stratified for three days in darkness in 0.1 % agar at 4°C and then grown *in vitro* on half-strength MS agar plates (9 x 9 cm). For all other biological stress experiments, seeds were surface sterilised, stratified for three days in darkness in 0.1% agar at 4°C and then germinated and grown on wetted rock-wool blocks (5x5x5 cm) supplemented with plant nutrition solution Hyponex (http://www.hyponex.co.jp/en/). Plants were grown in a climate chamber (16h light/8h dark, 24 ℃). Three week old plants were used to test the responses to different stresses including mechanical damage, JA, SA, spider mites, thrips and aphids or were left non-infested. The third leaf of each plant was chosen for treatments. Mechanical damage was made by six penetrations using a needle (outer diameter=0.2 mm) at the tip of the selected leaf. For JA and SA treatment, 5 µl 1 mM (+0.01% Tween 20) JA or SA respectively, was pipetted on the selected leaf. For herbivore infestations, five herbivores (either spider mites, thrips or aphids) were individually transferred from the rearing plants to the test plants using a fine brush with soft hair. We visually checked whether spider mites stayed on the leaf where they were introduced, during each of the light periods. Plants with mites that walked around the whole plant from the beginning of the experiments onwards, were discarded from the experiment. In experiments with aphids or thrips, each rock wool block with a plant was placed on a Petri dish (5 x 5 cm) in a tray filled with water (water level was lower than the petri dish) in such a way that Petri dishes did not touch each other to prevent herbivores moving to other plants. After luminescence measurements were ended, we visually checked for plant damage and whether herbivores were alive.

Trypan blue staining, luciferase measurements and GUS-staining

Trypan blue staining was performed according to a protocol described previously (Keogh et al., 1980). Leaves were immersed in lactophenol with trypan blue [10 ml lactic acid (Sigma L-1250), 10 g phenol, 10 ml glycerol, 10 ml water, 0.02 g trypan blue, diluted 1:2 v/v with ethanol before use] for one minute at 100 °C and left overnight in the staining solution.

Leaves were transferred to chloral hydrate solution for destaining for 2 days and observed under a stereo microscope. Photographs were made using a canon Power shot SX120 IS digital camera connected to the stereo microscope.

Analysis of Arabidopsis leaf luminescence was performed as described previously(Van Leeuwen et al., 2000) with some modifications. Plants were sprayed with 1 mM luciferin (1 mM firefly D-luciferin, 0.01% [v/v] Tween 20) until the whole plant was wetted but no drops fall down at 2 days and 8 hours in advance to stress treatments. In experiments that lasted more than three days, luciferin was re-sprayed every 3 days. Luminescence images of tested plants were acquired using a Pixis 1024B (1,024 x 1,024) camera system via a 35-mm, 1:1.4 Nikkor SLR camera lens (Nikon, Japan). A DT Green filter ring (Image Optics Components Ltd.) was fitted to the lens to block delayed auto-fluorescence emissions from chlorophyll. The whole camera system was connected to a computer and operated by MetaMorph microscopy automation and image analysis software (Molecular Devices Corporation, USA). For single time assays, plants were placed in complete darkness for 1 minute to decay auto-fluorescence and then imaged once with 7 minutes exposure. In experiments where dynamic responses were monitored, light was supplied from above the plants. Lights off or on and desired light/dark periods were automatically controlled by the same MetaMorph software. Luminescence images were automatically taken every hour during the time span of the assay. In the dark period the image was taken directly. In the light period, light was turned off for one minute to decay auto-fluorescence immediately before image acquiring during 7 minutes of exposure in darkness. Acquired images were analysed using ImageJ software (http://rsbweb.nih.gov/ij/). The interested regions (treated area, leaf or whole plant) were selected and the signal intensities were calculated using the function of “Measure” (for single measurement) in “Analyze” or function of “Measure stack” (for image sequence) from a plugin. Photon emission was depicted with false colour scales with blue indicating low activity and red indicating high activity.

GUS staining was performed according to a modified protocol from Hopkins et al. (Hopkins et al., 2009). A whole plant was immersed in staining solution [50 mM NaPO4 (pH 7.2), 2 mM X-gluc, 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6] and vacuum infiltrated for 30 min followed by overnight incubation at 37 ℃. After removal of the staining solution the sample was washed twice with tap water. Subsequently, samples were decoloured in increasing concentrations of ethanol (30%, 70%, and 96% each for one hour). Images were taken by a canon Power shot SX120 IS digital camera under bright field or a CCD camera connected to a microscope (Nikon, Optiphot-2, Japan).

Cucumber volatiles collection and analysis

Cucumis sativus 9930 (Chinese Long) plants were grown *in vitro*. Headspace samples were collected and analysed as described by Kappers et al. (2010) with adaptations. In brief, a four-week-old plant was enclosed in a glass jar (2.5 L) and air was drawn through a cartridge containing 200 mg of Tenax TA (20/35-mesh, Alltech) via an inlet with a flow rate of 150 ml min^{-1} to purify the incoming air. Volatiles emitted by the plant were trapped on a similar cartridge at the outlet that was interchanged automatically every four hours for a period of 6 days in total. Plants were placed in a climate chamber with a photoactive radiation (PAR) of 100 $\mu mol.m^{-2}.s^{-1}$ at the level of the plant and 22°C during the light period (12 hours) and 18°C during the dark period (12 hours). Twenty spider mites were introduced to the plant four hours after the start of the second light period. Collected volatile samples were dry purged under a stream of nitrogen for 10 minutes and analysed as described by (Kappers et al., 2010) on a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, MA, USA) quadrupole mass spectrometer.

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Supplementary data

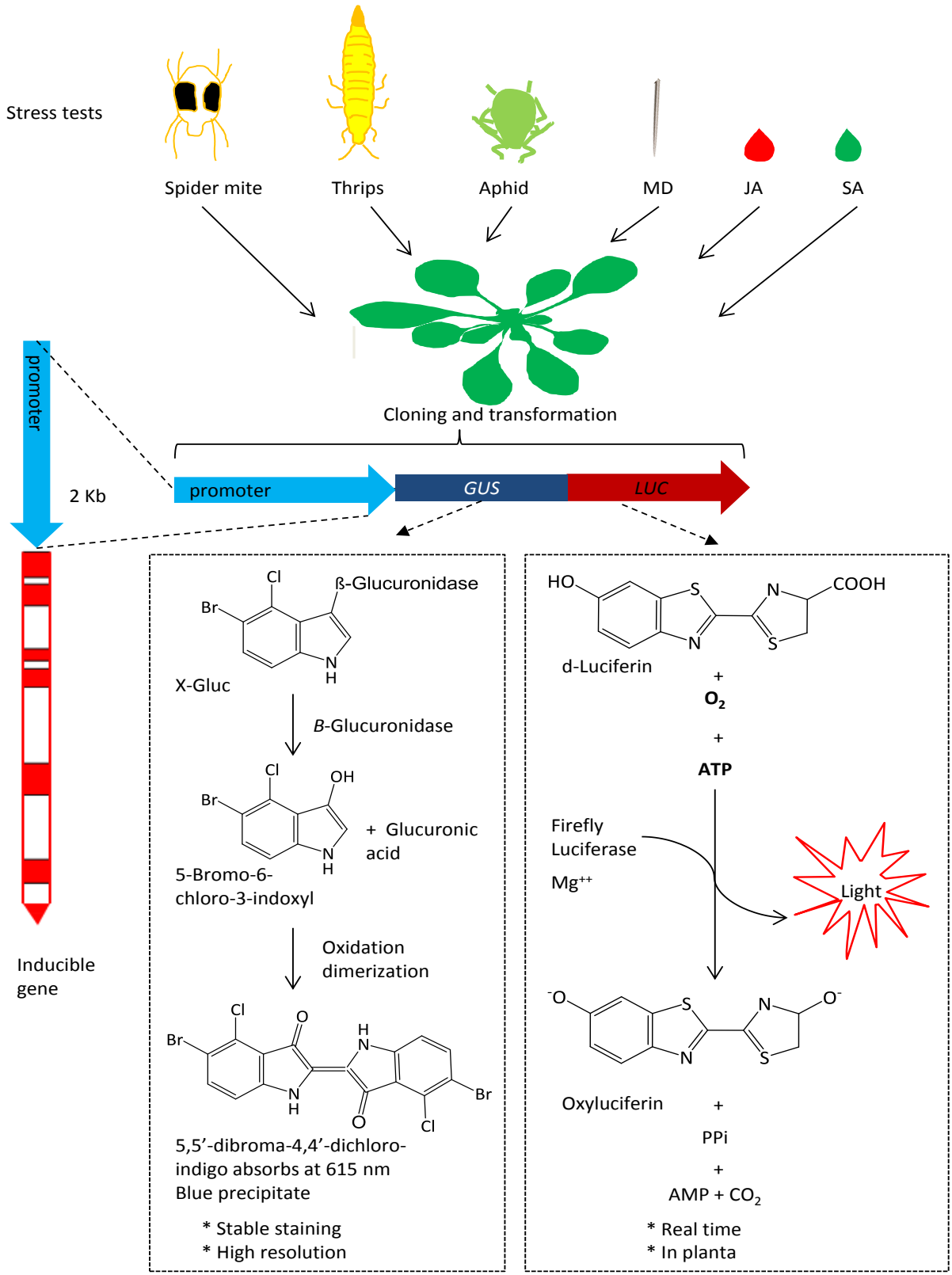

Fig. S1. Schematic representation of the experimental set-up. The 2 Kb sequence upstream of the ATG start codon was cloned in front of a GUS/LUC fused reporter gene and introduced into Arabidopsis thaliana. Transgenic Arabidopsis plants were challenged with different stresses and the promoter activities were analysed using GUS-staining or luminescence.

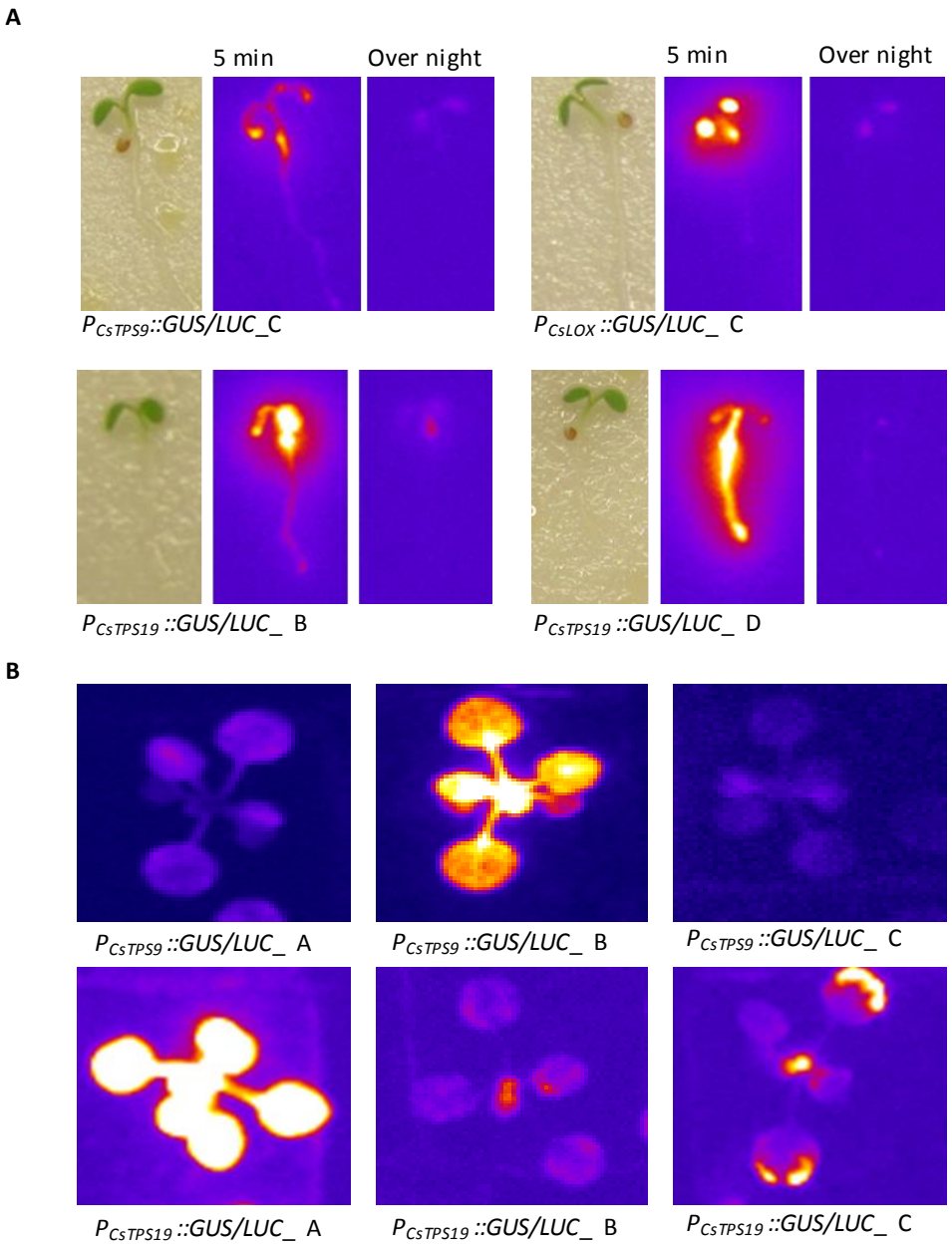

Fig. S2. Basal activities of promoters in non-stressed transgenic Arabidopsis plants. (**A**) Luminescence images of five-day old seedlings. (**B**) Luminescence images of three weeks old plants of different transgenic lines for *P_{CsTPS9}::GUS/LUC* and *$P_{CsTPS19}$::GUS/LUC*.

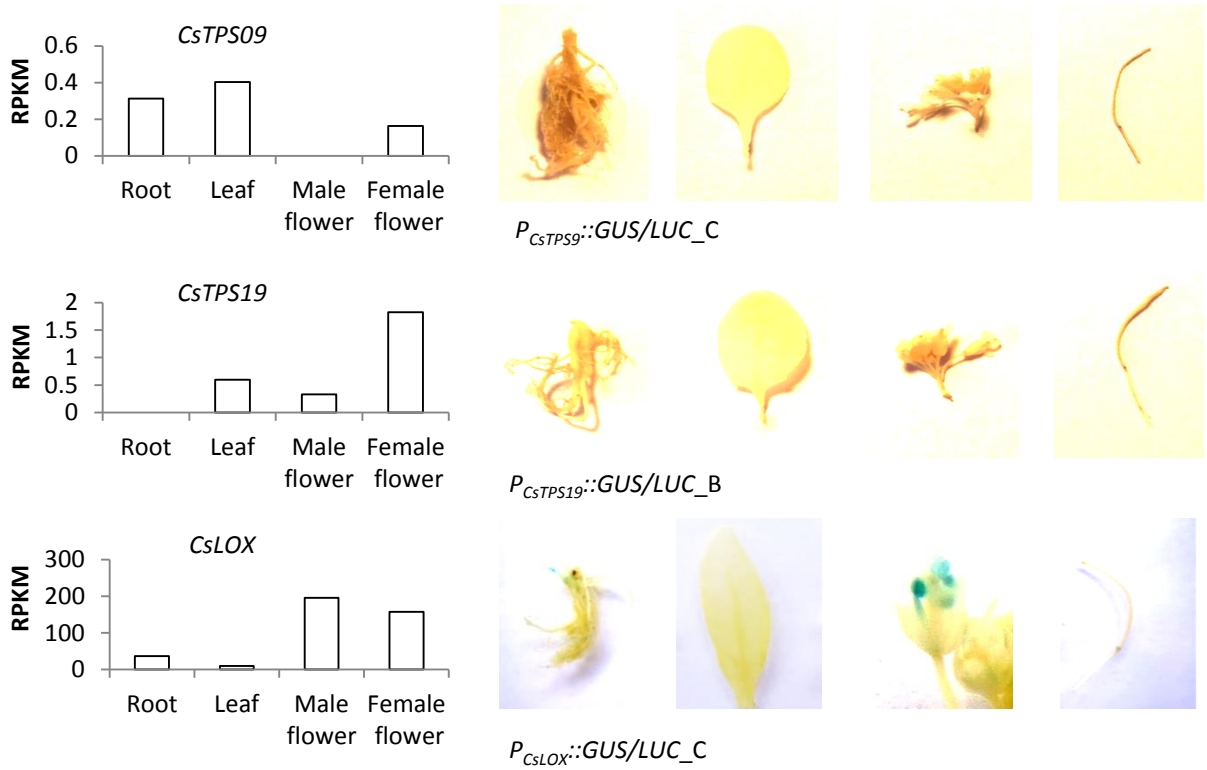

Fig. S3. Transcripts abundance of the selected genes in various cucumber organs analysed by RNA –seq [left, (Li et al., 2011)] and GUS-staining of different organs of non-stressed transgenic Arabidopsis of plants harbouring the corresponding promoters (right).

Chapter 6

General discussion

Jun He

To survive and reproduce within an environment with various organisms that attack them, plants must defend themselves from herbivory. Evolution has conferred the successful survivors with multiple mechanisms of defence. Plants may possess physical or chemical barriers which can interfere with the attack of certain herbivores. These defences can be constitutively present or be induced in response to herbivory. Inducible defence processes depend on signalling networks including recognition of attack, signal transduction, reconfiguration of the transcriptome and subsequently, for example, changes in the metabolome or morphology. In this thesis, I present an overview of multiple aspects of the inducible defence of cucumber plants that were infested by the two-spotted spider mite *Tetranychus urticae*. The effect of spider-mite feeding on induced defence in cucumber was studied at different levels including the genome, transcriptome and metabolome by using different ~omics and molecular biology technologies. The main aim of the thesis was to gain insight into the molecular mechanisms underlying cucumber induced defence in order to yield a knowledge base to support breeding for cucumber cultivars with improved indirect defence and that hence could result in more efficient biological control.

Using cucumber and Arabidopsis as model system to study plant-herbivore interactions with chelicerate spider mites

Domesticated cucumber (*Cucumis sativus*) and the two-spotted spider mite (*T. urticae*) were chosen as model in this study for both economical and scientific reasons. Economically, cucumber is the fourth most consumed vegetable and its annual world gross production value reaches more than 35 billion US dollar (http://faostat3.fao.org/home/E, final 2013 data). Up to 65% of the world production comes from China, followed by Japan (5%), Ukraine (4%) and Turkey (3%). Until 2013, the annual gross production value of cucumber in the Netherlands reached 302 million US dollar and comprised about 1% of the world gross production value. The primary pests in greenhouse cucumbers are western flower thrips [*Frankliniella occidentalis*, Shipp et al. (2000)], greenhouse whitefly [*Trialeurodes vaporariorum*, Boukadida and Michelakis (1994)] and the two-spotted spider mite [*T. urticae*, Park and Lee (2005)]. Breeding for cucumber cultivars with enhanced defence to these herbivores would benefit the environment and production of cucumber by decreasing the use of pesticides. A better understanding of the various defence mechanisms of cucumber against herbivory could help cucumber resistance breeding. In my thesis, I focused on defences to two-spotted spider mites that can reproduce very quickly and hence cause large economic damage to cucumber, especially in greenhouse production systems.

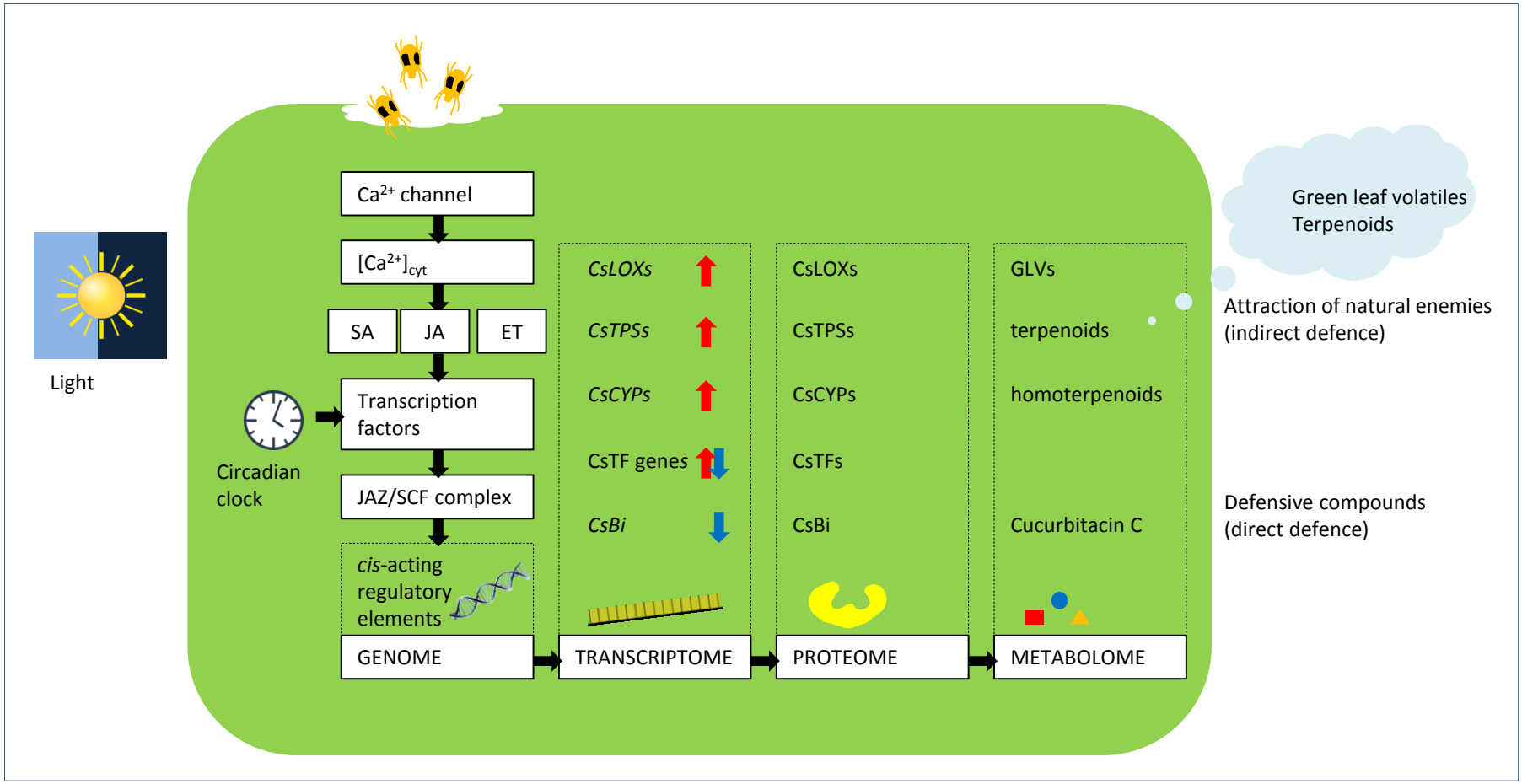

Fig. 1. Model of the induction mechanisms of defence responses of cucumber to chelicerate two-spotted spider mites including examples of genes, proteins and metabolites that are discussed and studied in this thesis. Black arrows indicate signalling or information cascades and red and blue arrows indicate up – and down-regulated gene expression upon spider-mite infestation. Abbreviations: SA: salicylic acid; JA: jasmonates; ET: ethylene; JAZ: jasmonate ZIM-domain; SCF: Skp/Cullin/F-box complex; LOX: lipoxygenase; TPS: terpene synthase; TF: transcription factor, Bi: BITTER (CUCURBITADIENOL SYNTHASE); GLVs: green leaf volatiles.

A direct defence mechanism in some cucumber genotypes is the presence of cucurbitacins, tetracyclic triterpenoids that are extremely bitter and are reported to have a negative impact on the feeding behaviour of multiple herbivores, including lepidopteran larvae, beetles and vertebrate grazers, including humans (Metcalf and Lampman, 1989, Metcalf et al., 1980, Tallamy and Krischik, 1989, Tallamy et al., 1997). In contrast, specialist herbivores like spotted and striped cucumber beetles feed intensively on bitter cucumber plants (Haynes and Jones, 1975). Cucurbitacin C was reported to be negatively correlated to oviposition by spider mites (Balkema-Boomstra et al., 2003) and spider-mite herbivory on cotyledons resulted in increased cucurbitacin production in the first systemic leaf (Agrawal et al., 1999). However, spider mites were reported to adapt within a few generations to cucurbitacins (Gould, 1979, Agrawal, 2000). Indeed, the expression of nearly half of the P450 genes in the spider-mite genome changes in response to feeding on different hosts, indicating a high adaptation potential to metabolise host anti-feedant compounds (Grbic et al., 2011). Recently, several genes encoding enzymes in the biosynthetic pathway of cucurbitacin C in cucumber were identified including seven *P450s*, the *CUCURBITADIENOL SYNTHASE* gene (*Bi*) and *ACYLTRANSFERASE* [*ACT*, Shang et al. (2014)]. In my study all these genes were downregulated upon herbivory by spider mites as demonstrated by RNA-seq and qRT-PCR (Chapter 2). Moreover, the content of cucurbitacin C also decreased in the spider-mite infested cucumber leaves (Fig. 1).

In addition to direct defence, the blend of volatiles emitted by cucumber plants upon herbivory by spider mites may play a role in indirect defence against spider mites by attraction of the natural enemies of this herbivore (Mercke et al., 2004, Kappers et al., 2010, Kappers et al., 2011). Although in this thesis I did not test the attractiveness of the volatiles from infested cucumber plants to predators, it was shown before that induced volatiles from spider-mite infested cucumber can attract the natural enemies of spider mites, e.g. predatory mites such as *Phytoseiulus persimilis*, to the infested plants (Takabayashi et al., 1994, Kappers et al., 2010, Kappers et al., 2011). I analysed the spider-mite induced volatile blend in detail and showed it consists mainly of three terpenes that were mostly only produced after herbivory (Chapter 4). Since terpenoids are important components in the herbivore-induced volatiles, in this thesis I identified the entire terpene synthase (*TPS*) gene family through analysis of the sequenced cucumber genome (Huang et al., 2009, Woycicki et al., 2011). I characterized the genes and the enzymatic activity of the corresponding proteins with particular attention for those that seemed to play a role in the formation of spider-mite induced terpenoid volatiles (Chapter 4).

The choice for cucumber to serve as a model species to investigate interactions with spider mites has a major obstacle as it is difficult to transform and hence it is not easy to characterize the function of genes using genetic tools such as overexpression or knockout. Therefore, we used *Escherichia coli* and *Arabidopsis thaliana* as heterologous expression

systems to investigate the function of cucumber genes. The *CsTPS* genes were isolated from cucumber and cloned into expression vectors and then individually expressed in *E. coli,* and the proteins were extracted and subsequently assayed *in vitro* for their enzymatic functions. By doing this, we determined the specific catalytic function of each of the CsTPSs. *Arabidopsis thaliana* ecotype Col-0 was used to make transgenic lines harbouring promoter::reporter constructs and was subsequently tested for its response to spider-mite infestation and a number of other stresses. Although, a recently published report showed that Col-0 is not very susceptible to spider mites (Zhurov et al., 2014), in our study successful infestation of Col-0 by spider mites was observed, indicated by chlorosis and trypan-blue stained dead cells during the herbivory (Chapter 5). Moreover, introduced cucumber promoters successfully function in Arabidopsis, indicating that the endogenous Arabidopsis transcription factors (TFs) and other aspects of the signal transduction pathway are capable to translate the signal from spider-mite recognition to expression of defence-related genes.

Cucumber responses to infestation by two-spotted spider mites

Plant defence starts from sensing the presence of the herbivore. Physical damage in plants as caused by herbivores varies among herbivores with different feeding habits. Chewing herbivores such as caterpillars bite off tissues of plants and can cause significant physical damage while phloem-feeding herbivores make little physical damage. Cell-content feeders, including spider mites and thrips inflict moderate physical damage. When spider mites feed on cucumber leaves, they pierce the epidermis with their stylets and feed on the contents of the mesophyll cells (Park and Lee, 2002), inflicting physical damage and likely interacting with the host through elicitors [Fig. 1, Dicke et al. (1993)]. In the present study, physical wounding caused by spider mites was observed both in cucumber and Arabidopsis. This enables to discriminate the local and distal influence of feeding by spider mites as the tissues surrounding the feeding sites are still present and functional. Similarly, it was observed that thrips also inflicted visible mechanical damage on Arabidopsis leaves and successfully activated the expression of *GLUCURONIDASE (GUS)* and firefly *LUCIFERASE (LUC)* fused reporter genes driven by the promoter of *CsTPS9*, while aphids, as expected, made no visible damage and failed to trigger the promoter. This fits with the observation that plants may respond differentially in gene expression to herbivores with different feeding habits (Ehlting et al., 2008).

My study showed that the content of jasmonic acid (JA) significantly increased in cucumber leaves during herbivory by spider mites, while salicylic acid (SA) only experienced slight changes (Fig. 1, Chapter 2). In consensus with this, the genes associated with JA, including genes encoding proteins involved in JA biosynthesis and genes known to be regulated by JA, were found to be upregulated upon infestation by spider mites while the SA-associated genes were not significantly enriched in the spider-mite regulated DEGs (Chapter 2).

Moreover, promoters of three individual genes, the spider-mite induced *CsTPS9*, *CsTPS19* and *CsLOX*, were only upregulated by application of JA but not SA (Chapter 5). These observations match each other and also support previous conclusions that JA plays a central role in the indirect defence of cucumber against spider mites (Bouwmeester et al., 1999, Mercke et al., 2004, Kappers et al., 2010). Application of JA could induce the release of a blend of volatiles from cucumber which contains many compounds that are also induced in response to feeding by spider mites and the JA-induced volatiles did attract natural enemies (Dicke et al., 1999, Bruinsma et al., 2009). In this thesis, I suggest that the JA-induced indirect defence is mediated through the transcriptional regulation of multiple genes (Chapter 2) and the JA-responsiveness of the promoters of the genes is associated with biosynthesis of terpenoids and green leaf volatiles in cucumber (Chapter 5).

Changes in the contents of phytohormones such as JA result in changes in the activities of transcription factors, for instance MYC2, MYC3 and MYC4 (Lorenzo et al., 2004, Fernandez-Calvo et al., 2011), that in turn regulate the expression of other genes. Global transcriptome changes of plants in response to herbivores have been documented for many species, for instance the response of rice to fall armyworm [*Spodoptera frugiperda*, Yuan et al. (2008)] and tomato to two-spotted spider mite (Kant et al., 2004, Martel et al., 2015). In particular, Arabidopsis has been analysed for transcriptome changes induced by herbivores including caterpillars, aphids and thrips (Reymond et al., 2004, De Vos et al., 2005, Kusnierczyk et al., 2007, Little et al., 2007). Transcriptome reconfiguration of Arabidopsis upon infestation by spider mites was studied using a microarray and more than 1000 genes were identified as differentially expressed during the first 24 hours of infestation (Zhurov et al., 2014). To date, only a few studies on the cucumber transcriptome have been reported. The transcriptome of a series of (un-stressed) cucumber organs was sequenced with the aim to improve the annotation of the putative gene set in the cucumber genome (Li et al., 2011). Although this study was not related to stresses associated with transcriptional responses, I used this dataset to find correlations between the diverse terpenoid volatiles emitted from different organs and the various expression patterns of the *CsTPS* genes. With regard to biotic stress, gene expression in cucumber leaves upon infection with downy mildew [*Pseudoperonospora cubensis,* Adhikari et al. (2012)] and gray mold [*Botrytis cinerea,* Kong et al. (2015)] was analysed. Previously the response of a number of cucumber genes to herbivory was studied (Mercke et al., 2004) and this thesis presents a genome-wide analysis of changes in gene expression in cucumber leaves upon herbivory by spider mites (Fig. 1). The 2348 DEGs triggered by spider mites at different time points after infestation likely include many genes that have a role in defence to spider mites. Screening the putative functions of these DEGs, we found that genes involved in the response to JA, genes encoding lipoxygenases, genes involved in photosynthesis and genes involved in terpene biosynthesis are induced by spider-mite infestation. Therefore, a solid platform was set up for further studies in mining key genes possibly conferring resistance of cucumber against cell content-feeding herbivores.

In my thesis, I had a more detailed look at three of these induced genes and studied the regulation of their promoters, which is discussed in more detail below.

As mentioned above, TFs play a crucial role in the translation of phytohormonal changes into defence responses. Among the 1212 identified TFs in the cucumber genome, about 10.3% were significantly regulated by spider mites. This portion of TF genes contains genes of various families including *MYB*, *bHLH*, *AP2/ERF* and *WRKY* as the four largest families. These TF gene families are known to be important in plant defence (Seo and Choi, 2015), although the expression profiles vary between the families and species studied. In spider-mite infested cucumber, *MYB*s and *bHLH*s were almost equally up- and downregulated, while *AP2/ERF*s and *WRKY*s were mostly upregulated after the introduction of spider mites (Chapter 3). The involvement of TFs in defence was reported for multiple TFs in many plant species. For example, AtMYB51, AtMYB75, PpMYB134, NtMYBJS1, NaMYB8 are associated with regulation of biosynthesis of various secondary metabolites which may function as defensive compounds (Gigolashvili et al., 2007, Borevitz et al., 2000, Mellway et al., 2009, Gális et al., 2006, Kaur et al., 2010). AtERF5, AtERF6, AtRAP2.2 were essential in defence responses to *B. cinerea* (Moffat et al., 2012, Zhao et al., 2012). WRKY3 and WRKY6 are capable of regulating defence in *Nicotiana attenuata* to herbivores (Skibbe et al., 2008). In our study, we also found that TFs were co-expressed with a number of genes that are associated with the biosynthesis of secondary metabolites (Chapter 3). For example, the TFs encoded by the genes which co-express with *CsTPS*s and *CsLOX*s putatively regulate the expression of these genes and eventually affect the biosynthesis and emission of terpenoids and green-leaf volatiles (Chapter 3). Future studies to elucidate these interactions could benefit from the dual GUS/LUC reporter system by e.g. *in vitro* or *in vivo* co-infiltration of TFs with the P_{CsTPS}*::GUS/LUC* constructs made in this thesis that would result in enhanced reporter activity in case of a successful interaction.

In consensus with the various TFs that were regulated in cucumber upon spider-mite infestation, analysis of the promoter regions of the DEGs showed the presence of various *cis*-acting regulatory elements (CAREs), in which I found an over-representation of binding motifs for MYB, bHLH, and AP2/ERF, TF families which were reported to be essential for plant defence (Lorenzo et al., 2004, Gigolashvili et al., 2007, Fernandez-Calvo et al., 2011) (Lu et al., 2011, Seo and Choi, 2015) and were suggested to be essential regulators for transcriptional changes in cucumber in response to herbivory of spider mites (Chapter 3). The presence of these CAREs in the promoters implies that the corresponding TFs can regulate the expression of the adjacent genes. The promoters of *CsTPS9* and *CsTPS19*, two genes strongly induced by spider mites, were also analysed for CAREs (Fig. 2). We found a number of different CAREs including multiple elements that could be involved in light regulated processes, defence to stresses, responsive to JA and SA, and also motifs that possibly are involved in circadian regulation. In agreement with this, P_{CsTPS9} and $P_{CsTPS19}$ in transgenic Arabidopsis showed responsiveness to light, upregulation by herbivory and by application of JA, while in contrast SA could not enhance promoter activity (Chapter 5).

After signal transduction and gene-expression changes, resistance of plants against herbivores can be achieved by physical alterations or changes in the form of metabolites. For instance, enhanced expression of genes involved in biosynthesis of indole glucosinolates in Arabidopsis resulted in an increase of these defensive metabolites and negatively influenced the feeding of spider mites (Zhurov et al., 2014). In contrast with the reported local and systemic upregulation of cucurbitacin C in cucumber plants which were infested by spider mites on the cotyledons (Agrawal et al., 1999), in our study we found that the genes involved in biosynthesis of the most well-known cucumber defensive metabolite cucurbitacin C (Shang et al., 2014) were down-regulated during the infestation by spider mites (Chapter 2). In agreement with the decreased gene expression, the content of cucurbitacin C in the spider-mite infested leaves also showed a decreasing trend during the first three days of infestation (Chapter 2, Fig. 1). These results suggest that spider mites suppress the biosynthesis of this toxic compound to facilitate feeding. Suppression of inducible defences by spider mites has been reported for a population of *T. urticae* in tomato plants (Kant et al., 2008) and a closely related spider-mite species, *T. evansi* reduced induced defence in tomato severely (Sarmento et al., 2011). However, further research will be required to fully understand what happens in cucumber. A possible approach would be to use elicitors from spider mites to trigger the defence response and analyse whether the amount of cucurbitacin C and the genes involved in its biosynthesis change in the absence of mechanical damage. Alternatively, spider mites adapted to bitter cucumber and spider mites adapted to non-bitter cucumber could be used to infest cucumber plants and to see if these spider mites cause difference in changes in cucurbitacin C. Now that both the genome of cucumber (Huang et al., 2009) and of spider mites (Grbic et al., 2011) are available, new possibilities arise to explore the plant-herbivore interaction on an integrative genomic level. For example, if and how the genome or transcriptome of the spider mites would change to adapt to accessions with or without cucurbitacin C during several generations.

We also studied genes that are responsible for changes in volatiles that are of importance for indirect defence. Spider-mite herbivory enhanced the emission of green-leaf volatiles and various terpenoids (Chapter 2, Chapter 4), as has been reported before (Bouwmeester et al., 1999, Mercke et al., 2004, Kappers et al., 2010, Kappers et al., 2011). These compounds have been demonstrated to be involved in the attraction of the predatory mite *P. persimilis* (Dicke et al., 1990, De Boer et al., 2004, Kappers et al., 2011) and thus may be important for the success of biological control strategies. As TPSs are essential to form the variable structures of terpenes, we paid special attention to these genes and studied the full gene family.

The putative *CsTPS* gene family in the cucumber genome was identified and contained 27 putative genes of which 19 are thought to encode complete functional enzymes. This CsTPS family is relatively small compared to the 40 TPSs in Arabidopsis (Aubourg et al., 2002), 44 in tomato (Falara et al., 2011) and 57 in rice (Goff et al., 2002). Similar to other plant species, most *CsTPS*s were organised in clusters, with three major clusters of which one is on

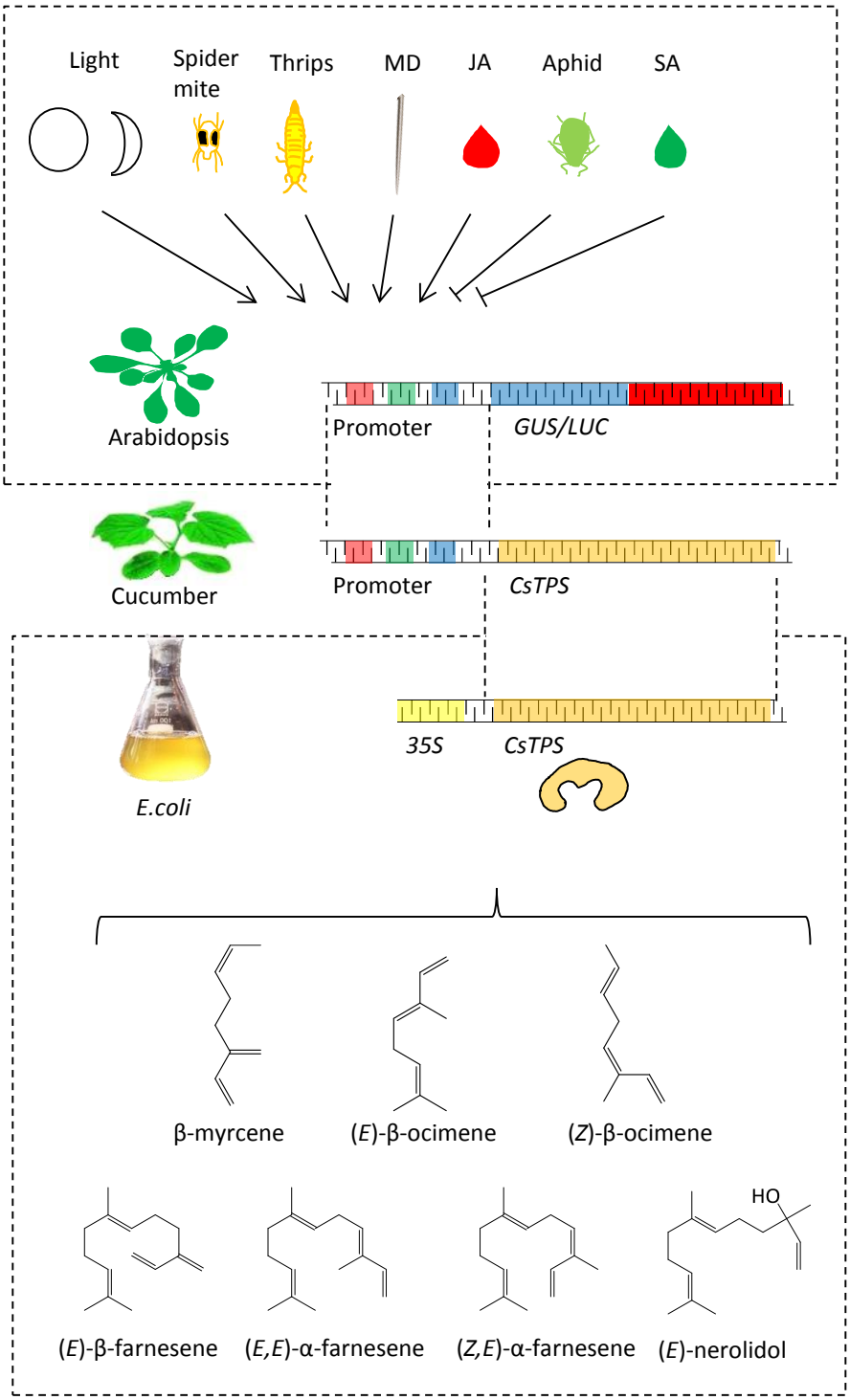

Fig. 2. The regulation and enzymatic function of cucumber gene *CsTPS9*. Black arrows indicate positive regulation. End locked lines indicate no regulation relationship found. Dashed arrows indicate detection approaches for reporters. Abbreviations: MD, mechanical damage; JA, jasmonic acid; SA, salicylic acid. GUS, β-Glucuronidase; LUC, luciferase; TPS, terpene synthase. GPP, geranyl diphosphate; FPP, farnesyl diphosphate; NPP, nerolidyl diphosphate.

chromosome II and two are on chromosome III. Multiple alignment of the sequences of *CsTPS*s showed that that the genes in the same clusters are more similar to each other than the genes outside the clusters, and this suggests a relatively recent duplication of the genes in these clusters. The presence of these genes in clusters may allow common regulation by chromatin modification (Wegel et al., 2009). In agreement with this, *CsTPS2* and *CsTPS3*, located in the same cluster, were both regulated during spider-mite herbivory. However, the other *CsTPS*s in the same cluster did not show co-expression with each other during infestation of spider mites.

The knowledge of which terpenes can be produced by the various CsTPSs is important to understand which genes are responsible for the volatile terpenes induced by herbivory. The CsTPSs together form the diversity of terpene backbone structures that can be produced in cucumber, under stressed or non-stressed conditions or in different developing stages and different organs. Although the *CsTPS*s comprise only a small gene family, the enzymes encoded by these genes can produce a wide variety of terpenes. In the enzymatic assays of the heterologously expressed proteins of the cloned 19 full length *CsTPS*s, multiple monoterpene and sesquiterpene products were formed from the same precursor geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), respectively (Chapter 4, Fig. 2). For example, CsTPS19 catalysed the formation of 15 different terpenoids from GPP and FPP. On the other hand, multiple CsTPSs produced the same products. For example, β-myrcene was produced by 13 different CsTPSs in our assays. Other terpenes were the result of enzymatic activity of a single CsTPS. For example, the structurally related β-pinene, α-pinene and sabinene were produced by CsTPS10 only.

Knowing the timing and localization of *CsTPS* expression is essential to understand the biological role played by these genes. The catalytic function of each of the CsTPSs determines the terpene potential while the expression level of each of the genes under specific conditions determines which of them are active. Although *CsTPS*s tend to be distributed in clusters within the genome, the expression of these genes varies among different cucumber organs as well as in response to infestation by spider mites. Some terpenoids (limonene, (*E*)-β-ocimene and linalool) are emitted in low amounts by leaves of non-infested cucumber plants and can be enhanced by spider-mite herbivory. Indeed the *LINALOOL SYNTHASES CsTPS1-3* and *(E)*-β-*OCIMENE SYNTHASE CsTPS5* are constitutively expressed in cucumber leaves and can be upregulated during infestation (Chapter 4). The presence of limonene in the emitted blend could be a side product of CsTPS3 activity. Other *CsTPS*s (e.g. *CsTPS9* and *CsTPS19*) are hardly expressed in non-challenged plants but are strongly induced upon herbivory. Indeed, multiple terpenoids were found only emitted by infested cucumber plants, including β-myrcene and (*E,E*)-α-farnesene which are products of CsTPS9 and CsTPS19. In total, the expression of six *CsTPS*s increased in cucumber leaves upon infestation by spider mites which is about one third of the functional CsTPSs. This implies that the rest of the CsTPSs may be responsive to other kinds of herbivores, or have a function in other organs or biological processes. For example, the higher content of linalool, (*E,E*)-α-farnesene, (*E*)-

nerolidol and τ-cadinol in female flowers than male flowers is in consensus with higher expression level of the genes encoding CsTPS19 which can form linalool and (*E,E*)-α-farnesene, CsTPS21 which can produce (*E*)-nerolidol, and CsTPS22 which can catalyse τ-cadinol in female flowers (Chapter 4), implying these expressed genes may play a role in pollinator attraction. Moreover, female and male flowers respond differentially to spider-mite feeding on the leaves (folivory), possibly caused by different expression patterns of the corresponding *CsTPS19, CsTPS21 and CsTPS22*. Similar to this in *Brassica nigra* plants, damage to the leaves by herbivores affects volatile emission in the flowers and makes the plants more attractive to pollinators than non-infested plants (Lucas-Barbosa et al. 2015). An interesting exception seems to be *CsTPS10*, which was not upregulated by spider-mite herbivory, but the terpenoids which the corresponding enzyme produces *in vitro*, α-pinene, α-phellandrene, sabinene and β-pinene, were emitted by cucumber leaves infested by spider mites (Chapter 4), implying that next to CsTPS other factor(s) may affect emission of terpenoids. *CsTPS*s may also possibly be involved in plant development. For example, *CsTPS23* encodes COPALYL DIPHOSPHATE SYNTHASE and *CsTPS24* ENT-KAURENE SYNTHASE, two proteins involved in gibberellin biosynthesis (Yamaguchi et al, 2008). High expression of these two genes in the ovary and low expression in the rest of the organs implies they may be involved in fruit developments (Talon et al, 1992; Fos et al, 2001).

TPSs are not the only important enzymes associated with terpenoid metabolism. The proteins associated with terpenoid precursor biosynthesis and the proteins associated with terpene modification also have essential influence on the emitted products. The genes encoding rate-limiting protein 1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE in the plastidial MEP/DOXP pathway which synthesize mono- and diterpenoid precursors were upregulated by spider-mite herbivory but the gene encoding the rate-limiting step 3-HYDROXY-METHYLGLUTARYL COENZYME A REDUCTASE in the cytosolic mevalonate pathway which produces sesqui- and triterpenoid precursors was downregulated upon spider-mite infestation (Chapter 2). Upregulation of MEP/DOXP is in consensus with the rapid and high induction of multiple monoterpenoids emitted by cucumber leaves in response to herbivory by spider mites (Chapter 4) and the downregulation of mevalonate pathway could be in consensus with the decrease in cucurbitacin C biosynthesis during infestation (Chapter 2). However, the production of several sesquiterpenoids, including (*E*)-β-caryophyllene and (*E,E*)-α-farnesene, from the same precursor pathway, was induced by spider-mite herbivory, in a time stage later than (*E*)-β-ocimene. Possibly, terpenoid precursors are exchanged between different subcellular compartments and/or preferentially allocated to specific pathways. Terpene structures made by TPSs can be modified further by other enzymes, such as CYTOCHROME P450s (CYPs), which belong to the superfamily of proteins containing a heme cofactor. In Arabidopsis CYP82G1 can convert nerolidol to (*E*)-DMNT [(*E*)-4,8-dimethyl-1,3,7-nonatriene] and geranyl linalool to (*E,E*)-TMTT [(*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene], two important herbivore-induced homoterpenes (Herde et al., 2008). In multiple plant genomes, *TPS* and *CYP* genes are located in clusters within a short distance of

each other (<200 Kb) (Boutanaev et al., 2015). The gene encoding CUCURBITADIENOL SYNTHASE, the first dedicated protein for the synthesis of the backbone of the triterpenoid cucurbitacin, was co-located with seven *CYP* genes and an *ACT* gene in the cucumber genome (Shang et al., 2014). However, the cucumber genome contains only a small number of these *TPS/CYP* gene pairs and the correlation of co-localization between both types of genes is not as significant as reported for other species (Boutanaev et al., 2015). One exception to this is the location of a gene encoding for a CYP adjacent to the *CsTPS* cluster on chromosome II. This protein belongs to the CYP78A class and is thus not an ortholog of AtCYP82G1, which is involved in DMNT and TMTT formation, as discussed above. I recorded that several *CYP* genes were upregulated by spider mites in the leaves of cucumber, while others were downregulated. The functions of these regulated *CYPs* are not clear, but the enzymes they encode could work together with CsTPSs to form the complex of terpene volatiles in response to herbivory. In particular, CYP enzymes most likely responsible for the conversion of nerolidol to (*E*)-DMNT and geranyl linalool to (*E,E*)-TMTT, are expected to be involved in and induced upon herbivory, as both these homoterpenes are present in the blend of spider-mite infested cucumber. To obtain this knowledge one needs co-assay the spider-mite induced CYPs with CsTPSs which can form nerolidol or geranyl linalool *in vitro*.

In my thesis I used promoter-reporter plants to study the regulation of gene expression of a number of genes in more detail. The expression of P_{CsTPS9} in transgenic Arabidopsis plants was induced by herbivory of cell-content feeding spider mites and thrips but not by phloem-feeding aphids and by JA but not SA (Fig. 2). Although introduction of a cucumber promoter in Arabidopsis is an artificial system, the activity of all three promoters investigated was induced by mechanical damage (Chapter 4) providing new evidence that mechanical damage is sufficient to activate the expression of *CsTPSs* and *CsLOX*, and these reactions could probably be generally shared in plants in response to any herbivores that inflict substantial mechanical damage. The enhanced luminescence as a result of thrips infestation was much stronger than that in response to spider-mite infestation and is in consensus with the more severe damage caused by thrips. The involvement of other factors like the oral secretions from thrips and spider mites in the activation of the promoters is suggested by the fact that luminescence triggered by spider mites increased 80-fold compared to non-infested plants within two days while mechanical damage only triggered luminescence to a maximum of about 4-fold. Although we were not able to distinguish the impact of the physical damage and the cues present in spider-mite saliva (Storms, 1971), our results suggest that the mechanical wounding is sufficient to trigger promoter activity, but other cues and/or repeated mechanical damage may be far more important than mechanical damage because the huge difference between the effect of mechanical damage and spider mites/thrips.

Luminescence of transgenic Arabidopsis plants harbouring P_{CsTPS9}*::GUS/LUC*, $P_{CsTPS19}$*::GUS/LUC* and P_{CsLOX}*::GUS/LUC* was different during light and dark periods, when these plants were under biotic stresses (Chapter 5). To study whether light dependency and or the circadian clock play a role in the activation of these promoters, transgenic plants were

challenged under different light/dark conditions (Fig. 2),. During three days of constant darkness, the activity of all three promoters disappeared while under continuous light luminescence was enhanced in a rhythmic pattern suggesting involvement of the circadian clock. A similar circadian rhythm was found for the *Artemisia annua* gene *QH6* encoding a monoterpene synthase (Lu et al., 2002, Zhou et al., 2015). Rhythmic emission of volatile metabolites was found in different species such as Norway spruce (*Picea abies*) induced by MeJA (Martin et al., 2003) or kidney bean (*Phaseolus vulgaris*) infested by the leafminer *Liriomyza huidobrensis*. Analysis of the emission of spider-mite induced volatiles from cucumber showed that emission of the green-leaf volatiles (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol and terpenes (*E*)-β-ocimene and (*E,E*)-α-farnesene was highest during light periods and much less during dark periods. The two terpene compounds in this blend are most likely the major products of CsTPS9 and CsTPS19, respectively (Chapter3).

Interestingly, the promoters of *CsTPS9* and *CsTPS19* displayed nocturnal activity while a diurnal emission was observed for the terpenoids synthesized by the corresponding proteins in cucumber leaves infested by spider mites. Light-dependent emission of (*E*)-β-ocimene was also found in lima bean plants (Arimura et al., 2008). When plants were damaged during the dark period low amounts of β-ocimene were emitted, and a burst of emission occurred after the onset of light (Arimura et al., 2008). High expression of *TPS* genes during the night can result in accumulation of the proteins in the plant leaves and hence enable the plants to immediately produce large amounts of terpenoid compounds when limiting factors such as precursor availability are relieved. It is reported that the MEP/DOXP pathway which produces the mono- and sesquiterpenoid precursors is regulated by light in many plant species (Hemmerlin et al., 2012). Although I did not study the expression of genes involved in the MEP/DOXP pathway during the light and dark periods, they are expected to be light dependent in cucumber as well. In conclusion, regulation of *CsTPS*s and genes of the upstream pathways by the circadian clock will eventually shape the diurnal emission of the terpenoid volatiles and result in a burst of emission during the light period, as observed before (Arimura et al., 2008) and in this study (Chapter 5).

Perspectives

In my thesis, I studied the molecular mechanisms underlying cucumber induced defence against spider mites. I studied how cucumber transcriptionally responds to herbivory by spider mites and which TFs are involved in the regulation of these genes. I identified the cucumber *CsTPS* gene family and characterized how the corresponding proteins form the blend of terpenoid volatiles emitted by cucumber (leaves or other organs, non-infested or infested). Finally, I demonstrated that promoters of some spider-mite inducible cucumber genes (*CsTPS9*, *CsTPS19* and *CsLOX*) are still inducible by herbivory of cell-content feeding

herbivores and JA after being introduced into Arabidopsis and show nocturnal activity while the terpenoid volatiles showed diurnal emission.

The knowledge gained from this thesis may be applied to crop breeding for enhanced resistance to herbivory, especially for cultivars with better indirect defence. We observed transcriptional regulation of a large number of genes in cucumber in response to herbivory by cell-content feeding spider mites. Identification of the key genes mediating the defence to herbivory would help breeders to focus on the right genes and/or markers. A possible approach to achieve this would be the analysis of the variation in expression of spider-mite regulated genes in the re-sequenced cucumber accessions (Qi et al., 2013). Together with volatile profiling upon herbivory this would give insight into which genes mediate the induced indirect defence in different cucumber cultivars. I analysed the TFs which are possibly involved in the regulation of gene expression in cucumber leaves during spider-mite infestation and pointed out the potential regulators for terpenoid and green leaf volatiles, and cucurbitacin C biosynthesis. These TF genes could include master regulators for the biosynthesis of these defensive secondary metabolites, and therefore could be used as marker genes for selection in breeding. My work also characterized the terpene product profiles of CsTPSs and expression profiles of corresponding genes on the family level, and this could explain most of the terpenoids that were present in the blend of induced or non-induced cucumber. This provides fundamental knowledge for breeding cultivars with unique terpenoid profiles upon herbivory. For instance, using a GMO approach one could engineer varieties with high emission of certain terpene volatiles by overexpression of the corresponding *CsTPS*s to make breeding material with high attractiveness to natural enemies, or more practically, via a non-GMO approach to select cultivars with special terpenoid profiles by checking the expression of the *CsTPS* genes in large germplasm collections. Moreover, cell-content-feeder inducible promoters were identified in cucumber, and these promoters could be used to engineer other non-induced (or even repressed, as I showed) defensive compounds, such as cucurbitacin C, to become inducible, locally expressed. Overall, this study will benefit the selection of cultivars with enhanced attractiveness to natural enemies of the pest species and improve biological control for greenhouse cucumber production.

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Summary

Plants have evolved mechanisms to combat herbivory. These mechanisms can be classified as direct defences which have a negative influence on the herbivores and indirect defence that attracts natural enemies of the attacking herbivores. Both direct and indirect defences can be constantly present or induced upon attack. Feeding by, and often even the presence of, herbivores can trigger a series of reactions in the plants including Ca^{2+} signalling, oxidative burst, changes in phytohormone levels, activation or suppression of transcription factors (TFs), regulation of gene expression and eventually re-configuration of the metabolome. An altered metabolome could result in enhanced defence in a direct way, as for example, defensive secondary metabolites that repel herbivores, or in an indirect way when new or more emitted volatiles attract natural enemies of the attacking herbivore to the infested plants. This study, using cucumber (*Cucumis sativus*) and the two-spotted spider mite (*Tetranychus urticae*) as model, aimed to reveal the molecular mechanisms underlying the induced defence during herbivory, with emphasis on transcriptional changes and the involved TFs, the enzymatic function of the genes associated with volatile biosynthesis, and their promoters which regulate their expression.

In **Chapter 2** genome-wide gene expression was studied in cucumber leaves during the early days of spider-mite herbivory using an RNA-seq approach. Up to 2348 cucumber DEGs (differentially expressed genes) were identified during the first three days of spider-mite herbivory. Functional analysis of the DEGs showed that these genes have a function in multiple biological processes including upregulated pathways such as the jasmonic acid (JA) signalling pathway, photosynthesis, biosynthesis of terpenoids, and downregulated pathways such as biosynthesis of the defensive secondary metabolite cucurbitacin C. In consensus with these transcriptional changes, chemical analysis showed that the JA content and emission of terpenoid volatiles increased while the content of cucurbitacin C decreased in cucumber leaves upon spider-mite herbivory.

To further understand the regulatory networks of inducible defence in cucumber towards spider mites, 1212 putative TF genes present in the cucumber genome were analysed, which showed that 119 were regulated by spider-mite herbivory (**Chapter 3**). According to the feature domains present on the corresponding proteins, these TF genes were classified into different gene families among which *MYB*s, *bHLH*s, *AP2/ERF*s and *WRKY*s were found to have the highest proportion of TF genes that were affected by the infestation of spider mites. Comparison of the TF genes regulated by spider-mite infestation and pathogen infection (downy mildew) revealed similarity as well as differences in the response of TF genes to both biotic stresses. Co-expression of the spider-mite affected TF genes with metabolite biosynthesis genes such as *CsTPS*s, *CsLOX* and genes involved in cucurbitacin C biosynthesis pointed to TFs which potentially play a role in the regulation of the pathways involved in the biosynthesis of these metabolites. Analysis of the CAREs (*cis*-acting regulatory elements) in the promoter regions of the DEGs showed an over representation of binding motifs for MYB,

bHLH and ERF/AP2 TFs, once more suggesting the essential role these TFs have in the regulation of gene expression in cucumber leaves upon spider-mite herbivory.

Terpenoids, next to green leaf volatiles, are major components present in herbivory-induced plant volatile blends and they are assumed to be essential cues for the attraction of natural enemies of herbivores. Therefore, the whole *CsTPS* gene family containing 27 members was annotated from the previously sequenced cucumber genome and they were found to be mainly distributed in four clusters located on three chromosomes (**Chapter 4**). Nineteen *CsTPS*s with a putative full-length sequence were isolated and 17 of them were successfully expressed in *Escherichia coli* and shown to encode enzymes that can convert the terpenoid precursors geranyl diphosphate and/or farnesyl diphosphate into various terpenoids. Up to 31 different terpenoids were produced by these CsTPSs when assayed *in vitro*. Analysis of the expression of the *CsTPS*s in cucumber organs and in cucumber leaves upon spider-mite herbivory revealed that their expression correlates with the emission of terpenoid volatiles in non-infested and spider-mite infested leaves, in male and female flowers of plants without and with folivory and in roots treated with JA.

To obtain insight into how inducible defence related genes are spatially and dynamically expressed in response to herbivory, the promoters of *CsTPS9* and *CsTPS19*, two genes associated with terpene volatile biosynthesis, and *CsLOX*, a gene possibly associated with green leaf volatile biosynthesis, were isolated from cucumber leaves, fused to a *GLUCURONIDASE/FIREFLY LUCIFERASE* dual reporter and subsequently introduced into *Arabidopsis thaliana* (**Chapter 5**). The promoter activities in response to different stresses were analysed in the resulting transgenic Arabidopsis plants. The promoters of all three genes were activated in response to spider-mite herbivory. The promoters of *CsTPS9* and *CsTPS19* showed enhanced activity in response to mechanical damage and application of JA, but not upon treatment with salicylic acid. Furthermore, the promoter of *CsTPS9* was activated upon herbivory by thrips (*Frankliniella occidentalis*), an herbivore of the same feeding guild as spider mites, but not by aphids (*Myzus persicae*) which feed on phloem sap and do not inflict visible damage. Dynamic measurements of the luminescence of transgenic Arabidopsis plants under regular and altered light/dark regime including continuous light and continuous darkness revealed that promoters of these volatile-associated genes have nocturnal activity which is likely regulated by the circadian clock. In contrast, dynamic collection and measurement of the volatiles released by cucumber plants infested with spider mites showed diurnal emission, implying that other factors such as the regulation of genes associated with terpenoid precursor biosynthesis and regulated by the circadian clock in an opposite way, may work together with CsTPSs to result in emission of terpenoid volatiles in cucumber foliage upon herbivory during the day.

Finally, in **Chapter 6** the work presented in this thesis and the molecular mechanisms underlying the induced defence responses in cucumber to herbivory by two-spotted spider mites are discussed. The different chapters on transcriptional profiling, transcription factors,

the genes they regulate and the promoters responsible for this regulation are connected. Finally, the possibilities to use the knowledge gathered in my thesis for breeding of cultivars with improved induced indirect defence and, hence, improved biological control, are discussed.

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About the author

Curriculum vitae

Jun He was born on the 24th of May 1982, in Sichuan, China. He got his bachelor degree in plant genetics and breeding at the China Agricultural University in Beijing, China. He did his master study in the Chinese Academy of Agricultural Sciences, also in Beijing, under the supervision of Prof. Dr Sanwen Huang. Here, he worked on non-host defence in Arabidopsis against potato late blight (*Phytophthora infestans*) for six months and subsequently he participated for two years in *de novo* genome sequencing of potato and cucumber in the Beijing Genomics Institute (BGI). After he obtained his master degree in 2008, he continued to work in BGI, as a researcher on the sequencing part of a comparative genomics project related to C4 rice research. From June 2011, he started as a PhD candidate at the Laboratory of Plant Physiology and Laboratory of Entomology, Wageningen University in the Netherlands. Under supervision of Prof. Dr Harro Bouwmeester, Prof. Dr Marcel Dicke and Dr Iris Kappers, he worked on molecular mechanisms underlying cucumber inducible defence to two-spotted spider mites, which is described in this thesis.

Publication:

Jun He, Harro J. Bouwmeester, Iris F. Kappers. Transcriptional changes in cucumber (*Cucumis sativus*) in response to two-spotted spider mite (*Tetranychus urticae*) infestation reveal induction and suppression of defence-related pathways. In preparation.

Jun He, Marcel Dicke, Harro J. Bouwmeester, Iris F. Kappers. Characterization of the terpene synthase family in cucumber. Submitted.

Jun He, Marcel Dicke, Harro J. Bouwmeester, Iris F. Kappers. Promoters of cucumber defence-related genes respond to herbivory, mechanical wounding and jasmonic acid in a light-dependent rhythm in transgenic Arabidopsis with a GUS/LUC fused reporter system. In preparation.

Participated in:

Huang, S.W., et al., The genome of the cucumber, *Cucumis sativus* L. Nature Genetics, 2009. 41(12): p. 1275-1281.

Xu, X., et al., Genome sequence and analysis of the tuber crop potato. Nature, 2011. 475(7355): p. 189-195.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Jun He**
Date: **8 April 2016**
Group: **Laboratories of Plant Physiology and Entomology**
University: **Wageningen University & Research Centre**

1) Start-up phase		*date*
►	**First presentation of your project**	
	Work plan: Genome Mining for Enhancing Biological Control in Cucumber	Nov 07, 2011
►	**Writing or rewriting a project proposal**	
►	**Writing a review or book chapter**	
►	**MSc courses**	
►	**Laboratory use of isotopes**	
	Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure		*date*
►	**EPS PhD student days**	
	EPS PhD student day, University of Amsterdam	Nov 30, 2012
	EPS Get2Gether, Stayokay in Soest , NL	Jan 29-30, 2015
►	**EPS theme symposia**	
	EPS Theme 4 Symposium, Wageningen UR	Dec 09, 2011
	EPS Theme 2 Symposium, Wageningen UR	Feb 10, 2012
	EPS Theme 3 Symposium, Utrecht Universtiy	Apr 26, 2012
	EPS Theme 3 Symposium: Metabolism and Adaptation, University of Amsterdam	Mar 22, 2013
►	**Annual Experimental Plant Sciences meeting (Lunteren) and other National Platforms**	
	Annual Meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2012
	Annual Meeting 'Experimental Plant Sciences', Lunteren	Apr 22-23, 2013
	Annual Meeting 'Experimental Plant Sciences', Lunteren	Apr 15, 2014
	Annual Meeting 'Experimental Plant Sciences', Lunteren	Apr 13-14, 2015
►	**Seminars (series), workshops and symposia**	
	Attendance seminars of Invited lecturers	2011-2014
	Chinese PhDs Meeting	Sep 20, 2011
	Symposium: Parasitic plants and strigolactones	Oct 07, 2011
	ExPectationS (EPS Career Day), Wageningen	Nov 18, 2011
	Workshop on plant-insect interactions, University of Amsterdam	Nov 23, 2011
	Symposium Plant Breeding in the Genomics Era, Wageningen	Nov 25, 2011
	Sequencing seminars	Dec 07, 2011
	ServiceXS Symposium	Dec 08, 2011
	ExPectationS (EPS Career Day): Creativity and inspiration in science , Wageningen	Feb 01, 2013
	Workshop on plant-insect interactions, Wageningen UR	Sep 24, 2013
	Workshop on plant-insect interactions, Utrecht University	Nov 03, 2014
	Plant Sciences Seminar on Bioinformatics, WUR	Nov 04, 2014
	Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding (0.3)	Dec 11, 2014
	Workshop Last Stretch of the PhD Programme	May 22, 2015
►	**Seminar plus**	
►	**International symposia and congresses**	
	PhD Retreats: 6th European Plant Science Retreat in Amsterdam, the Netherlands	Jul 01-04, 2014
►	**Presentations**	
	Poster: Cloning and Characterization of the Terpene Synthase Family in *Cucumis sativus*, Lunteren	Apr 22-23, 2013
	Poster: Cucumber Terpene Synthases Genes Induced by Spider Mites, Lunteren	Apr 13-14, 2015
	Talk: STW project progress presentation (STW user committee meeting), ENZA zaden, Enkhuizen	Jun 12, 2012

	Talk: STW project progress presentation (STW user committee meeting), ENZA zaden, Wageningen	Dec 06, 2012
	Talk: STW project progress presentation (STW user committee meeting), ENZA zaden, Wageningen	Mar 31, 2015
	Talk: Transcriptomal responses of bitter and non-bitter cucumber to infestation by the cell-feeding spider mite *Tetranychus urticae*; ALW meeting 'Experimental Plant Sciences', Lunteren, NL	Apr 15, 2014
	Talk: Transcriptomal changes in cucumber (*Cucumis sativus* L.) in response to infestation with spider mites (*Tetranychus urticae* Koch), 6th EPSR, Amsterdam, NL	Jul 01-04, 2014
	Talk: Terpene synthases in Cucumber, PII, Utrecht University	Nov 03, 2014
	Talk: Terpene synthases genes induced by spider mites in cucumber (PhD trip,Zürich, Switzerland)	Apr 29, 2015
►	**IAB interview**	
	Meeting with a member of the International Advisory Board of EPS	Jan 05, 2015
►	**Excursions**	
	PPH PhD trip (Germany, Switzerland)	Apr 22-May 01, 2015
	Subtotal Scientific Exposure	*21.7 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Systems Biology: 'Statistical Analysis of ~Omics Data'	Dec 10-14, 2012
	An Introduction to Mass Spectrometry-based Plant Metabolomics	Dec 09-13, 2013
	Transcription Factors and Transcriptional Regulation	Dec 17-19, 2013
►	**Journal club**	
	Participation in the plant physiology literature discussion group	2011-2015
►	**Individual research training**	
	Subtotal In-Depth Studies	*6.9 credits**

4) Personal development		*date*
►	**Skill training courses**	
	PhD Competence Assessment	May 22, 2012
	Course: Presentation Skills	Sep 12 & 26, 2012
	Course: Practical English Plus	Sep 2012-Feb 2013
	Course: The Essentials of Scientific Writing and Presenting	Dec 2013
	Course: English for IELTS	Aug 18-29, 2014
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*6.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**36.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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