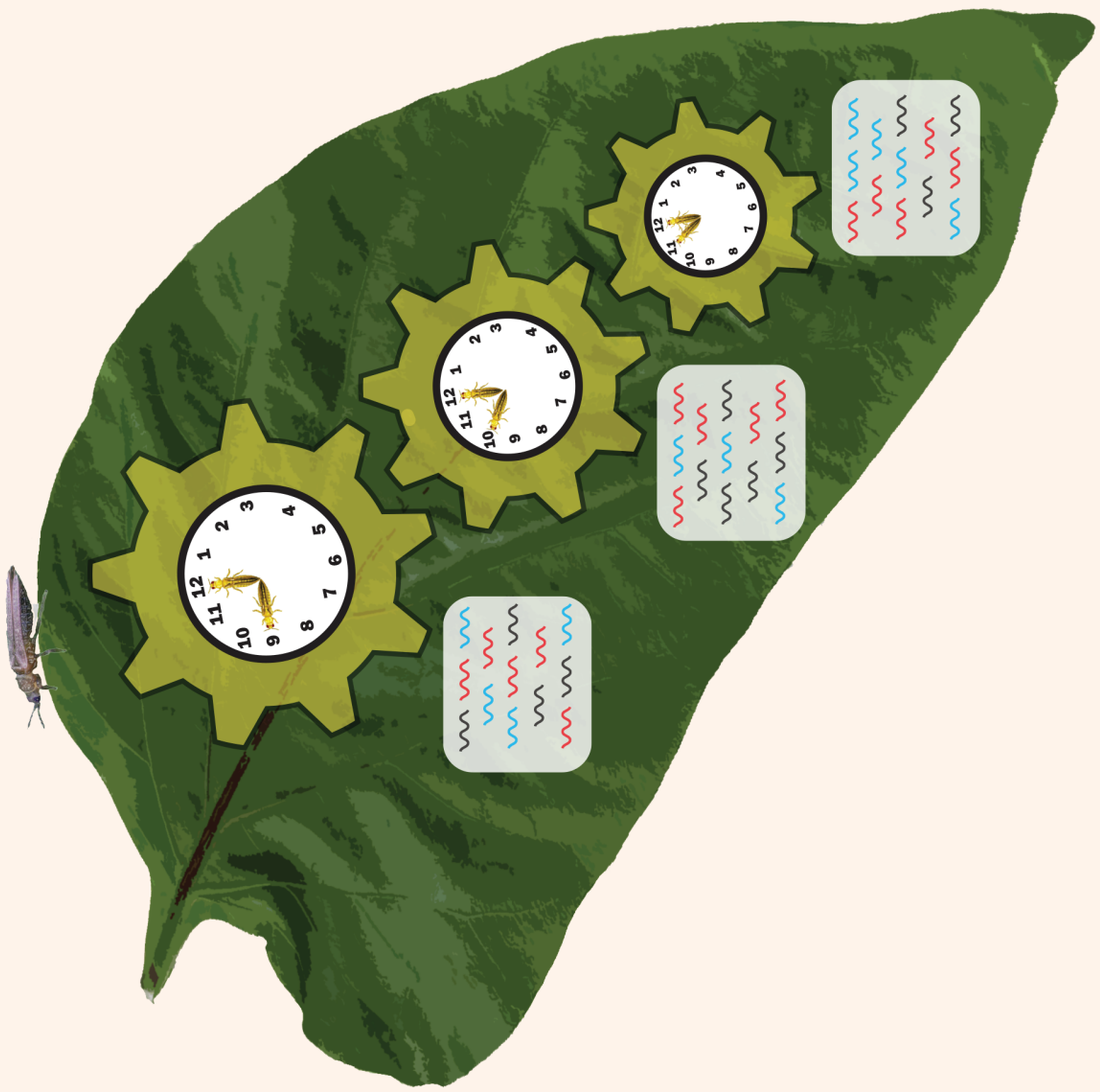
Dynamics of transcriptional responses of plants to thrips feeding



Sandeep J. Sarde

Dynamics of transcriptional responses of plants to thrips feeding 2019 Sandeep J. Sarde

Propositions

1. Transcriptional responses of plants have more similarities in upregulated genes than in downregulated genes.
   (this thesis)

2. Translation of research from model to non-model plants is a myth.
   (this thesis)

3. In science, analytical advances are more needed than technological advances.

4. We are sinking in data and starving for knowledge.

5. Nature has its own programming script.

6. To live in dignified poverty is better than to live in undignified wealth.

7. Accomplishment is in pursuing excellence and not success.

Propositions belonging to the thesis entitled,

“Dynamics of transcriptional responses of plants to thrips feeding”

Sandeep J. Sarde

Wageningen, 20th June 2019

Dynamics of transcriptional responses of plants to thrips feeding

Sandeep J. Sarde

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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences

Dynamics of transcriptional responses of plants to thrips feeding

Sandeep J. Sarde

Thesis

submitted in fulfilment of the requirements for the degree of doctor
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by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
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Sandeep J. Sarde

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To my beloved family,

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Chapter 1

General introduction

Plants and herbivorous insects

Interactions between plants and herbivorous insects have existed for more than 400 million years (Labandeira, 2007). To combat herbivorous insects, plants have evolved two layers of defences, constitutive and induced defences. Constitutive defences are always present in the plants, whereas induced defences are elicited in response to external stimuli such as chemical, physical or (a)biotic stresses. Induced defences can be further categorized into direct and indirect defences. Direct defences are plant traits that influence the performance of herbivorous insects (Howe & Jander, 2008; War *et al.*, 2012). In contrast, indirect defences promote the enemies of herbivores, e.g. by the release of a cocktail of volatile compounds, termed volatile organic compounds (VOCs) or herbivore-induced plant volatiles (HIPVs), that attract natural enemies (predators and parasitoids) of the attacking herbivorous insects (Dicke, 2009; McCormick *et al.*, 2012; Mithofer & Boland, 2012; Dicke, 2015).

Furthermore, plants perceive herbivorous insects via damage-associated molecular patterns (DAMPs) and herbivore associated molecular patterns (HAMPs) (Mithofer & Boland, 2008; Heidel-Fischer *et al.*, 2014; Duran-Flores & Heil, 2016; Hilker & Fatouros, 2016). The perception of herbivores can depend on, 1) mode of insect feeding and 2) the specific effectors, derived from insect saliva or eggs (Heidel-Fischer *et al.*, 2014). Effector proteins are produced by herbivorous insects in their saliva that can interfere with and regulate the induced plant defences to the benefit of the herbivores (Hogenhout & Bos, 2011; Kant *et al.*, 2015; Giron *et al.*, 2016). Moreover, based on the mode of feeding, herbivorous insects can broadly be classified into three major groups, 1) chewers like caterpillars, 2) sap feeders like aphids and whiteflies and 3) cell-content feeders like spider-mites and thrips (Stam *et al.*, 2014). Ample knowledge on different elements of induced plant defences such as HAMPs (Douglas, 2018), effector proteins and resistance genes (Hogenhout & Bos, 2011; Reymond, 2013; Douglas, 2018) is available for leaf chewers and phloem feeders, but not for cell-content feeders.

Induced plant defences against herbivores

Early recognition of an attacking herbivore can be a key factor for a plant's success in defending itself. Induced plant defences against herbivores can be categorized into three main phases: recognition, signalling and response (Heidel-Fischer *et al.*, 2014). At the plant-insect interface, plants recognize herbivorous insects through specific insect-inflicted damage or insect-associated specific chemical cues (Mithofer & Boland, 2008; Heidel-Fischer *et al.*, 2014; Duran-Flores & Heil, 2016; Hilker & Fatouros, 2016). Subsequent to herbivore recognition, a series of early and late (in timescale of seconds to days) events occurs in plants (Maffei *et al.*, 2007). The earliest mechanism (timescale of seconds to minutes) is an alteration in plasma membrane potential

(V_m), followed by variation in cytosolic Ca^{2+} concentrations and the biosynthesis of H_2O_2 (Maffei *et al.*, 2007). Consequently, at a timescale of minutes, kinases and phytohormones are induced, altering the expression of transcripts (timescale of minutes to hours) and the biosynthesis of metabolites (timescale of hours to days) (Maffei *et al.*, 2007; Stam *et al.*, 2014). The temporal rearrangements of the transcriptome form the basis of extensive changes occurring in plant phenotype through the activation and deactivation of various biological processes (Stam *et al.*, 2014). This dynamic change in phenotype can influence the entire ecology of plants, throughout the season (Poelman *et al.*, 2010) or over different seasons (Stam *et al.*, 2018) and plant interactions with insects at different trophic levels (Stam *et al.*, 2014).

Plant hormones, such as jasmonic acid (JA), ethylene (ET) and salicylic acid (SA), are major players in regulating defences in response to feeding by different insect herbivores (Pieterse *et al.*, 2009; Verhage *et al.*, 2010; Pieterse *et al.*, 2012; Stam *et al.*, 2014). For example, JA especially mediates responses induced by chewing insects like caterpillars (Reymond *et al.*, 2004; De Vos *et al.*, 2005) and cell-content feeding insects like thrips (Abe *et al.*, 2008; Abe *et al.*, 2009; Steenbergen *et al.*, 2018), whereas SA mediates responses to phloem-feeding insects like aphids and whiteflies (Zhu-Salzman *et al.*, 2004; Walling, 2008; Pieterse *et al.*, 2012; Tzin *et al.*, 2015; Broekgaarden *et al.*, 2018). Ethylene (ET) often acts synergistically with JA to fine-tune induced plant defences against herbivorous insects (Pieterse *et al.*, 2009; Pieterse *et al.*, 2012; Stam *et al.*, 2014). Besides, other plant hormones such as gibberllins, abscisic acid (ABA), cytokinins and auxins are known to play a role in responses to herbivory. The regulation of induced defences to herbivory by these plant hormones extensively shapes the plant phenotype.

Transcriptional responses of plants to herbivore feeding

Several studies have captured whole-genome transcriptional responses of *Arabidopsis* against different insect herbivores using microarray or RNA-Seq platforms. Whole-genome microarray studies have been performed in response to caterpillars (Reymond *et al.*, 2004; De Vos *et al.*, 2005; Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011; Zhang *et al.*, 2013; Appel *et al.*, 2014; Coolen *et al.*, 2016; Davila Olivas *et al.*, 2016; Kroes *et al.*, 2017), whiteflies (Kempema *et al.*, 2007; Zhang *et al.*, 2013) and aphid infestation (De Vos *et al.*, 2005; Bidart-Bouzat & Kliebenstein, 2011; Kroes *et al.*, 2017). Reymond *et al.* (2004) reported that 67-84 % of the total transcriptional response of *Arabidopsis* to *Pieris rapae* feeding is regulated by JA. Similarly, microarray or RNA-Seq platforms were used to capture whole-transcriptome profiles of non-model plant species in response to feeding by different insect herbivores. For instance, wild cabbage response to caterpillars (Broekgaarden *et al.*, 2011b) or whiteflies (Broekgaarden *et al.*, 2018), maize response to aphids (Tzin *et*

al., 2015) and tomato, maize, barley and grapevine to spider mites (Martel *et al.*, 2015; Diaz-Riquelme *et al.*, 2016; Bui *et al.*, 2018). The limitation of these studies on different insect herbivores is that the plant transcriptional response assessment is based on one or two time points over a period of 24 hours or longer (De Vos *et al.*, 2005; Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011; Zhang *et al.*, 2013; Appel *et al.*, 2014; Diaz-Riquelme *et al.*, 2016; Kroes *et al.*, 2017; Broekgaarden *et al.*, 2018; Tu *et al.*, 2018) with the exception of a study that comprised six time points for the response of wild tobacco plants to *Manduca sexta* oral secretion application, over a time span of 13 hours (Durrant *et al.*, 2017).

Several recent studies have shown that a plant dynamically reconfigures its transcriptome with time (Breeze *et al.*, 2011; Windram *et al.*, 2012; Hickman *et al.*, 2017). However, the transcriptional responses captured in response to different insect herbivores present low-resolution portraits of transcriptional responses, as the studies were performed with limited time points (one or two time points in a period of 24 hours or longer) (De Vos *et al.*, 2005; Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011; Zhang *et al.*, 2013; Appel *et al.*, 2014; Diaz-Riquelme *et al.*, 2016; Kroes *et al.*, 2017; Broekgaarden *et al.*, 2018; Tu *et al.*, 2018). Therefore, generation of high-resolution time-series transcriptional data in response to insect herbivory will result in a more detailed understanding of the temporal transcriptional reprogramming of plants. Thorough quantification of transcripts over several time points can elucidate the chronology of biological pathways (phytohormones, secondary metabolites or developmental processes), involvement of major transcription factors (TFs) and building a platform to construct gene regulatory networks. In addition, performing comparative transcriptomics using the high-resolution transcriptional data of different plant species in response to insect herbivory can identify commonalities and specificities of induced responses on plant-species or plant-family level. Moreover, this comparison can also unravel other aspects, such as relative response time of different plant species, complexity of transcriptional response and order of changes in biological events.

JA has been identified as a central player in modulating defences against several arthropod herbivores (Pieterse *et al.*, 2009; Verhage *et al.*, 2010; Pieterse *et al.*, 2012; Stam *et al.*, 2014). Lipoxygenases (LOXs), a multi-gene family, are known to be involved in several developmental and defence-related biological processes such as fruit ripening, tuber development, seed germination and JA-regulated plant defences (Kolomiets *et al.*, 2001; Bailly *et al.*, 2002; Feussner & Wasternack, 2002; Kessler, 2004; Barry & Giovannoni, 2007; Yan *et al.*, 2013). Upon feeding by many insect herbivores, LOXs catalyse oxygenation of polyunsaturated fatty acids (PUFAs) to initiate the formation of hydro-peroxides such as oxylipins (Shibata & Axelrod, 1995; Brash, 1999; Feussner & Wasternack, 2002). Several forms of oxylipins such as jasmonates, green leaf volatiles (GLVs) and death acids, are known to be involved in

defence mechanisms against insect herbivory (Bell *et al.*, 1995; Allmann *et al.*, 2010; Yan *et al.*, 2013; Shen *et al.*, 2014; Christensen *et al.*, 2015; Losvik *et al.*, 2017). Furthermore, based on positional specificity to catalyse oxygenation of linoleic acids (LAs), LOXs are broadly classified into two major groups, 9- and 13-LOXs (Feussner & Wasternack, 2002). Jasmonates and GLVs, involved in direct and indirect defences, are derived from 13-LOXs, whereas, death acids are derived from 9-LOXs. Furthermore, different numbers of *LOXs* are reported in different plant species such as Arabidopsis (6) (Umate, 2011), tomato (7), kiwifruit (6) (Zhang *et al.*, 2006), olive (4) (Padilla *et al.*, 2009, 2012), melon (18) (Zhang *et al.*, 2014), cucumber (23) (Liu *et al.*, 2011) and grapevine (18) (Podolyan *et al.*, 2010). Among the *LOXs* in each plant species, one *LOX* is involved in JA-biosynthesis induced upon insect herbivory or mechanical wounding. For example, *AtLOX2* in *Arabidopsis* (Bell *et al.*, 1995), *SlLOXD* (*TomLOXD*) in tomato (Yan *et al.*, 2013), *NaLOX3* in tobacco (Halitschke & Baldwin, 2003; Kessler, 2004) and *StLOXH3* in potato (Royo *et al.*, 1996) are known to code for enzymes involved in JA biosynthesis. Therefore, to understand the mechanism of JA-regulated defences, identifying the *LOX* involved in JA pathway is important.

Thrip pests

Thrips (Paraneoptera: Thysanoptera), are pest insects on many commercial and ornamental plants worldwide. The order Thysanoptera represents over 5500 species. Thrips are tiny (ca 1.5 mm) cell-content feeding insects exerting direct damage to plants by inserting their stylets into plant tissue and ingesting the cell contents. The pierced tissue turns into silvery scars (Fig. 1C) hindering photosynthetic ability, growth, reproduction and yield of plants (Steiner, 1990; Welter *et al.*, 1990; Shipp *et al.*, 1998; Steenbergen *et al.*, 2018). The success of thrips as a pest can be attributed to several of its characteristics, such as its short life-cycle, high polyphagy, thigmokinetic behaviour, high reproductive rate, and rapid adaptation to insecticides (Diaz-Montano *et al.*, 2011; Gill *et al.*, 2015; Steenbergen *et al.*, 2018).

Besides inflicting direct damage, thrips also cause indirect damage by serving as vector to different tospoviruses. For example, western flower thrips (WFT; *Frankliniella occidentalis*) is known to transfer *Tomato spotted wilt virus* (TSWV) (Maris *et al.*, 2003), whereas onion thrips (*Thrips tabaci*) is known to transfer *Iris yellow spot virus* (IYSV) (Bunyaviridae) (Diaz-Montano *et al.*, 2011; Gill *et al.*, 2015). Moreover, although western flower thrips is reported to be one of the economically most damaging species, several other thrips species, such as onion thrips, avocado thrips (*Scitothrips perseae*), blossom thrips (*Frankliniella schultzei*) and melon thrips (*Thrips palmi*) also contribute significantly to overall economic losses. The estimation of losses caused only by thrips feeding is hard, but the crop losses caused due to combined effect of thrips feeding and transferring the viral disease is huge. At present, thrips are mostly

controlled by using pesticides. Therefore, to develop thrips-resistant crop varieties to minimize the damage, exploration and understanding of the genetic basis underlying plant defence responses is important. Such crop varieties may be a valuable component of integrated pest management, in combination with biological control (Mouden *et al.*, 2017).

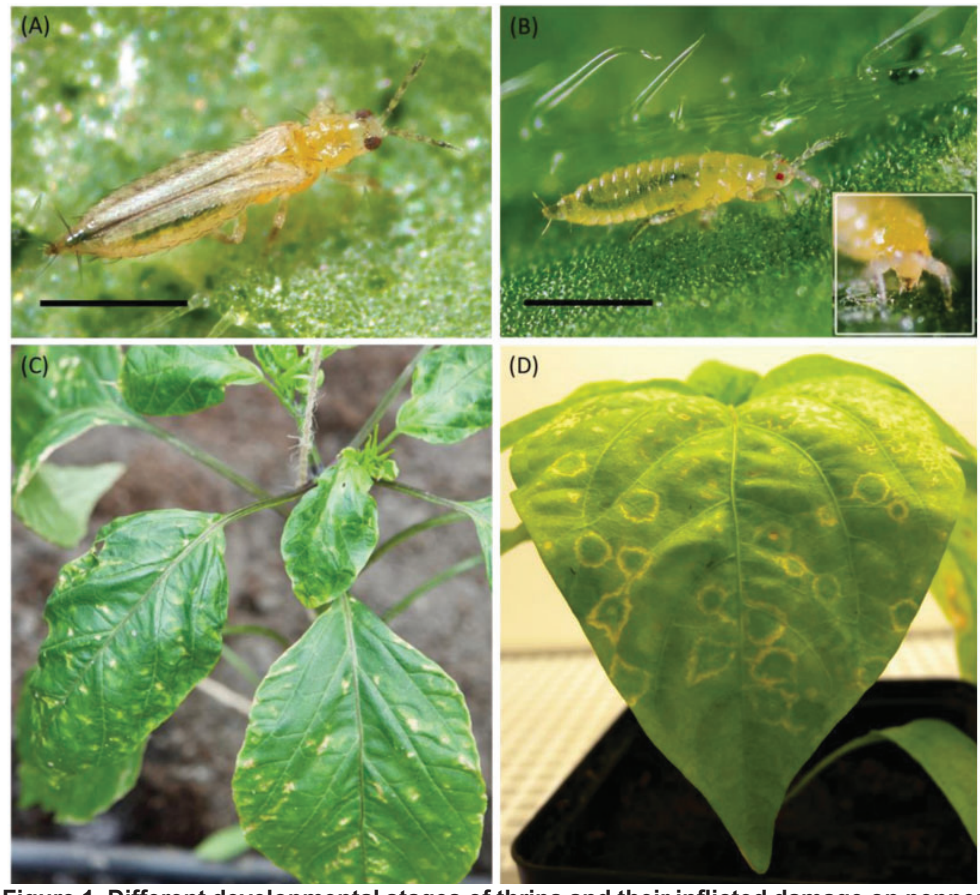

Figure 1. Different developmental stages of thrips and their inflicted damage on pepper plants. (A) Female adult *Frankliniella occidentalis* (Western flower thrips (WFT)), (B) second larval instar (L2) of WFT, (C) Silver scars resulting from feeding by thrips, (D) Symptoms of *Tomato spotted wilt virus* (TSWV). Adapted from Steenbergen *et al.* (2018).

Thrips: Feeding behaviour and virus transmission

Feeding behaviour

For a thorough understanding of how thrips damage plants, it is important to investigate their feeding behaviour in detail. Thrips feed on almost all aboveground parts of plants: leaves, flowers, fruits and stems. While feeding, they insert their stylets into

the tissue of a plant and ingest cell contents (Hunter & Ullman, 1992; Mound, 2005). During individual probes they can ingest cell contents from multiple cells (Kindt *et al.*, 2003). Probing comprises of three distinct behavioural phases: (1) piercing into plant parts (2) salivation and (3) ingestion of cell contents. When feeding on leaves, thrips can only access cell contents of epidermal, mesophyll and parenchymal cells, since their stylets are too short to reach vascular tissues.

Kindt *et al.* (2003) characterized feeding behaviour of *F. occidentalis* into six different phases: P, Q, R, S, T and U using the electrical penetration graph (EPG) technique (Kindt *et al.*, 2003; Kindt *et al.*, 2006). These phases captured all minor behavioural activities of thrips during feeding, where each phase is represented by one waveform. Waveform P depicts piercing of the leaf surface by the mandibular stylets, waveform Q represents maxillary stylet insertion and the beginning of salivation and waveform R represents ingestion of the cell contents. Finally, waveforms S and U represent the mandibular action and waveform T represents withdrawal of stylets from leaf. Since not all these waveforms occur during every probe event, Kindt *et al.* (2006) proposed to discriminate the waveforms into two major phases, i.e. “puncture phase” (P, Q, plus S) and “feeding phase” (R, T, plus U) (Kindt *et al.*, 2003; Kindt *et al.*, 2006). Thus, with EPG and histological studies, details of the exact feeding mechanism of thrips as mediated by its stylets within the plant tissues have been elucidated.

Virus transmission by thrips

Besides direct damage, thrips also cause damage to plants indirectly by transmitting plant viruses like Tospoviruses, among which TSWV, that have significant economic impact (Maris *et al.*, 2003; Steenbergen *et al.*, 2018). Tospoviruses represent the only plant-infecting genus in the family Bunyaviridae, infecting thousands of plant species (Whitfield *et al.*, 2005). In order to gain broad knowledge on how thrips damage plants indirectly, understanding of acquisition and transmission of viruses is important.

Acquisition of tospoviruses by thrips is dependent on thrips developmental stage. Viruses are generally acquired by immature first (L1) and second (L2) larval instars of thrips while feeding on viruliferous plants. The efficiency of acquiring viruses is higher at first instars (L1) and decreases as they develop (Rotenberg *et al.*, 2015). The acquired viruses then replicate in the host vector and are transmitted to healthy plants thereafter (De Assis *et al.*, 2004). The transmission of TSWV can occur as fast as in a single non-ingesting probe. This transmission of TSWV occurs during the salivation phase, i.e. Q-waveform of thrips feeding (Kindt *et al.*, 2003). The success rate of transmission of TSWV by *F. occidentalis* in a thrips-TSWV susceptible variety was reported to be 0.8 %, i.e. one in 125 probes resulted in successful transmission of virus (Kindt, 2004). The probability of virus transmission by a viruliferous thrips increases with increasing numbers of probes. Likewise, the efficiency of transmission

of viruses largely depends on the plant variety the thrips are feeding on. For example, virus inoculation efficiency was significantly lower on a thrips-resistant pepper accession during longer inoculation periods but no effect was seen in shorter inoculation periods (Maris *et al.*, 2003). This indicates that varieties resistant to thrips can provide opportunities to control virus spread as well.

Host-plant resistance to thrips

Host-plant resistance to insects is an important component of integrated pest management (Sharma & Ortiz, 2002). Understanding the mechanisms underlying genetic variation in resistance within a population of accessions can help to develop host-plant resistance against insects in a crop (Broekgaarden *et al.*, 2011a). However, linking genetic variation to specific traits is a complex task, due to the involvement of the number of traits underlying plant-insect interactions. Host-plant resistance is regulated by a complex network of genes. As a consequence, the development of a reproducible high-throughput method providing information on different components of host-plant resistance is important (Kloth *et al.*, 2012). At present, there are two major methods to determine host-plant resistance to thrips: (1) end-point assays, and (2) detailed observations of insect behaviour. In end-point assays, thrips performance is measured based on damage, mortality and reproduction on the host-plant, whereas, in behavioural assays, insect preference during the period of an experiment is recorded (Thoen *et al.*, 2016). In end-point assays, host-plant resistance to thrips has been established from days to weeks post-inoculation. For this type of assays, thrips damage is assessed manually or automatically by using imaging tools. Additionally, other parameters of insect performance like survival, reproduction and mortality have also been recorded manually (Abe *et al.*, 2008; Abe *et al.*, 2009; Maharijaya *et al.*, 2011; Leiss *et al.*, 2013). Such assays are labour intensive and time consuming. Thoen *et al.* (2016) developed a high-throughput phenotyping platform that can screen host-plant resistance against *F. occidentalis*, where they considered thrips behaviour as a proxy of resistance. The method consists of multiple, simultaneous two-choice setups using computerized continuous video-tracking of thrips behaviour throughout a period of several hours. In this method, detailed behavioural parameters like time spent on either accession, movement (searching), non-movement (feeding) are recorded and analysed. With a higher time and resource efficiency, this method complements the results produced in end-point assays (Thoen *et al.*, 2016). Although these two approaches produce quite different information on host-plant resistance against thrips, combining these methods can be used to efficiently find the factors responsible for host-plant resistance against thrips. The automated video-tracking method has as major advantage that hundred plants can be screened simultaneously per day. Thus, this method can be used to screen hundreds of plant samples for narrowing the selection, whereas end-point assays can be used to validate the selection made by the

video-tracking method. Recently, the video-tracking system is updated to automation (known as T-maze arrays) making it less laborious and time-consuming (Jongsma *et al.*, 2019). Therefore, this method can be a reliable and effective high-throughput phenotyping alternative to the behavioural assays.

Induced plant defences upon thrips feeding

Upon thrips feeding, plants trigger signal transduction pathways that regulate transcriptomic responses and biosynthesis of several processes, such as metabolites and defence-related proteins. The plant hormone JA is identified as a pivotal hormone in regulating induced defence against thrips (Fig. 2) (De Vos *et al.*, 2005; Abe *et al.*, 2008; Abe *et al.*, 2009). In plants, such as, *Arabidopsis* and Chinese cabbage (*Brassica rapa subsp. pekinensis*), WFT feeding elevated the expression of JA-biosynthetic genes and JA hormonal levels (Abe *et al.*, 2008; Abe *et al.*, 2009). *Defenceless1 (Def1)*, a JA-deficient tomato mutant, has reduced resistance to thrips feeding (Li *et al.*, 2002; Escobar-Bravo *et al.*, 2017). A full-genome microarray-based study in *Arabidopsis* showed that 69 % of all differentially expressed genes (DEGs) upon thrips feeding were JA responsive (De Vos *et al.*, 2005). Exogenous application of JA to several plants, such as *Arabidopsis*, Chinese cabbage (*Brassica rapa subsp. pekinensis*), tomato, soybean and cotton, enhanced the resistance against thrips (Omer *et al.*, 2001; Thaler *et al.*, 2001; Li *et al.*, 2002; Abe *et al.*, 2008; Abe *et al.*, 2009; Selig *et al.*, 2016; Escobar-Bravo *et al.*, 2017).

Furthermore, plants produce defensive metabolites to defend themselves against herbivores. In response to thrips feeding, phenolic compounds are found to be produced by plants (Papadaki *et al.*, 2008; Leiss *et al.*, 2009; War *et al.*, 2012). For instance, during pepper (*Capsicum annuum*) - *F. occidentalis* and alfalfa (*Medicago sativa*) - *Odontothrips loti* interactions, levels of phenolic compounds like tocopherols and tannins were elevated, respectively, in resistant varieties of both plant species (Maris *et al.*, 2003; Wang *et al.*, 2014). In contrast, in pepper, thrips-induced metabolites such as alkanes and fatty acids correlated positively with susceptibility of pepper plants (Maharijaya *et al.*, 2012). Furthermore, few studies have shown plants to modify their blend of volatile organic compounds (VOCs) in response to thrips infestation and attract natural enemies of thrips, thus activating their indirect defences. For instance, upon WFT feeding, cucumber plants attracted the predatory mite *N. cucumeris* and predatory bug *Orius lavigatus* (Venzon *et al.*, 1999). Similarly, during eggplant - *T. palmi* and chrysanthemum (*C. morifolium*) - *F. occidentalis* interactions, infested eggplant (*Solanum melongena*) and chrysanthemum plants attracted *Orius sauteri* and *Neoseiulus cucumeris*, respectively (Manjunatha *et al.*, 1998; Maris *et al.*, 2003).

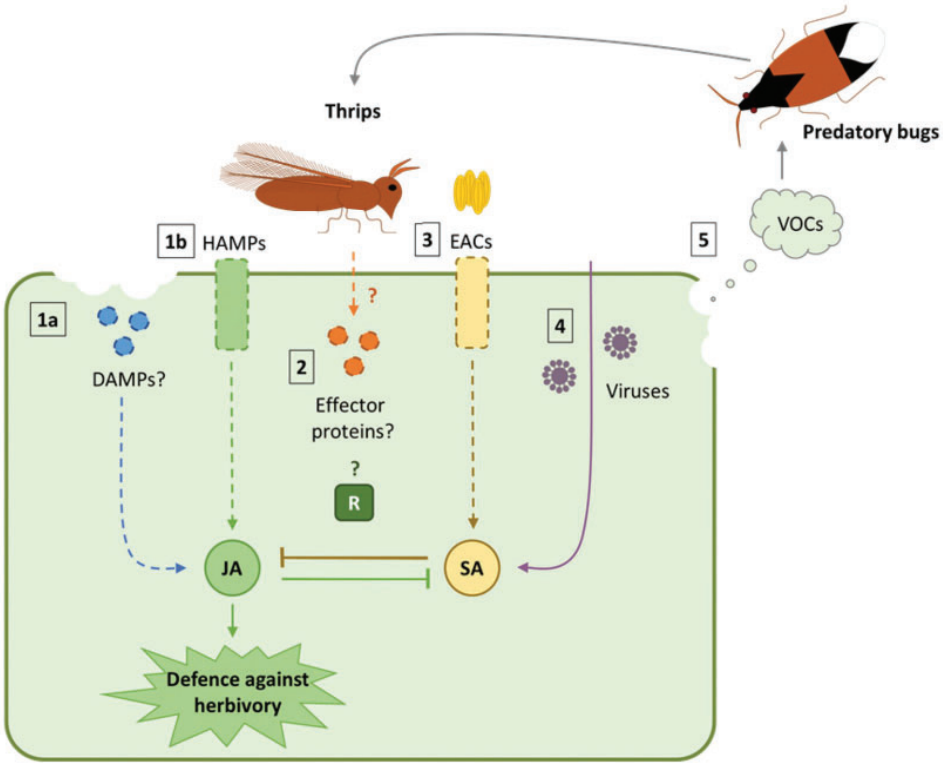

Figure 2. Proposed model of induced plant responses upon thrips feeding. During the plant-thrips interaction, plants initiate defence responses upon perceiving damage-associated molecular patterns (DAMPs) (1a) or herbivore-associated molecular patterns (HAMPs) (1b). This results into activation of JA, which further regulates the defences against thrips. Effector proteins (2) and egg associated compounds (EACs) (3) derived from thrips can manipulate induced plant defences in favour of thrips. Tospoviruses transmitted by thrips can alter plant defence by inducing SA, which in turn can supress JA-regulated responses (4) through cross-talk. Thrips feeding results in the release of a blend of VOCs that attracts natural enemies of thrips such as, *Orius laevigatus* (5). Dashed lines represent unidentified elements in induced responses against thrips. Adapted from Steenbergen *et al.* (2018).

Research objective

The objective of this thesis was to study the whole-genome transcriptional response of plants to thrips through a high-resolution temporal analysis and to investigate the genetic mechanisms activated in crop plants (sweet pepper and white cabbage) in response to thrips feeding. This includes identifying defence-related gene families, disentangling functions of genes and elucidating temporal whole-genome transcriptional response of sweet pepper and white cabbage against WFT and onion thrips feeding, respectively.

Study system

Plant species

1. Sweet pepper

Sweet pepper (*Capsicum annuum*) [(Mandy variety, Rijk Zwaan (De Lier, The Netherlands))] plants (Fig. 3A) were used to study the transcriptional response to western flower thrips feeding. Sweet pepper is a diploid and self-pollinating crop belonging to the Solanaceae family. This family encompasses several other commercially important crops such as eggplant, tomato, tobacco, potato and petunia. Pepper has a high nutritional quality and provides us with important minerals, vitamins and nutrients. It can be produced for several purposes such as, in pharmaceuticals, organic colour, cosmetics and defence repellents. However, in spite of the rising economic importance of pepper, information on the underlying molecular mechanisms against herbivorous insects including thrips is limited.

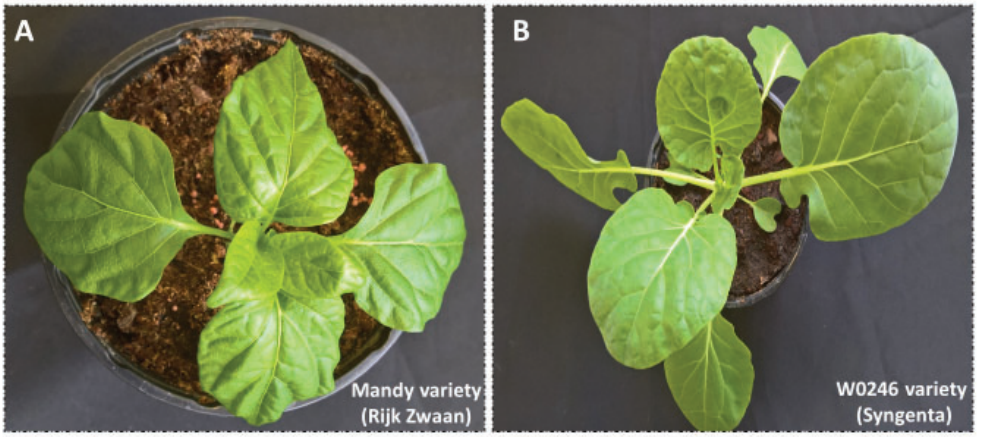

Figure 3. Sweet pepper and white cabbage plant varieties used in this thesis. (A) Sweet pepper (Mandy) and, **(B)** White cabbage (W0246) variety.

2. White-cabbage

White cabbage [*Brassica oleracea* (W0246 variety, Syngenta (Enkhuizen, The Netherlands))] plants (Fig. 3B) were used to investigate the temporal transcriptional response against onion thrips. White cabbage is an biennial plant grown as an annual, belonging to the Brassicaceae (Crucifer) family. *Brassica oleracea* comprises of several common food crops such as kale, cabbage, broccoli, cauliflower, Brussels sprouts and kohlrabi. The molecular mechanisms of white cabbage against insect herbivores have received limited attention.

Thrips species

1. Western flower thrips

Western flower thrips *(Frankliniella occidentalis)* (Fig. 4A) were used to infest sweet pepper plants (Chapters 1, 2 and 3 of this thesis). In The Netherlands, WFT is a major pest on sweet pepper plants in greenhouses. Thrips are cell-content feeding generalist insects affecting plant productivity and yield by inflicting feeding damage and transferring tospoviruses such as, TSWV. In several plants, such as *Arabidopsis*, Chinese cabbage (*Brassica rapa subsp. pekinensis*) and tomato, they are known to elicit JA-regulated defences (Abe *et al.*, 2008; Abe *et al.*, 2009).

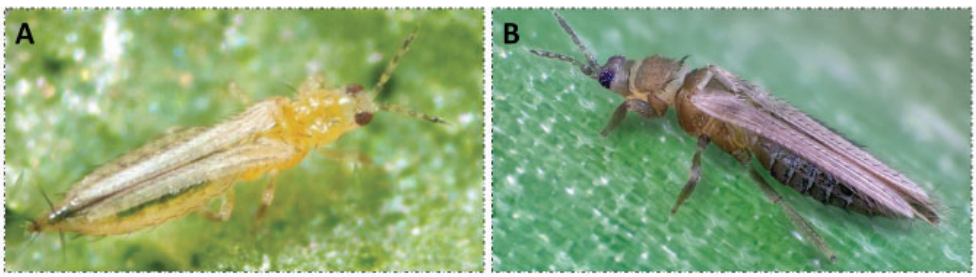

Figure 4. Thrips species used in this thesis. (A) Western flower thrips (*Frankliniella occidentalis*) interaction with sweet pepper and, **(B)** Onion thrips (*Thrips tabaci*) interaction with white cabbage. Photo credits: **A** modified from Steenbergen *et al.* (2018) and **B** adapted from Thrips-iD website: http://www.thrips-id.com/en/.

2. Onion thrips

Onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) (Fig. 4B), was used in combination with white cabbage plants in Chapter 4 of this thesis. This thrips is a serious pest on white cabbage (*B. oleracea*) plants worldwide (Shelton *et al.*, 2008; Fail *et al.*, 2013). It has similar life-history characteristics as WFT, making them difficult to control. Onion thrips transmits other tospoviruses than WFT, i.e. *Iris yellow spot virus* (IYSV) (Bunyaviridae) (Diaz-Montano *et al.*, 2011; Gill *et al.*, 2015). To the best of my knowledge, no molecular study has been made yet in any plant on the response to onion thrips.

Outline of the thesis

Chapter 2 focusses on the identification and classification of the lipoxygenase gene family in pepper. Implementing several approaches such as comparative genomics, domain-scan analysis, sequence analysis, phylogenetic analysis, homology modelling and transcriptional analysis, the lipoxygenase gene family of pepper (*Capsicum annuum*) was identified.

Chapter 3 focusses on the involvement of one *LOX* gene, i.e. *CaLOX2,* in jasmonate-dependent induced defence against western flower thrips in sweet pepper. With Virus-Induced Gene Silencing (VIGS) of *CaLOX2*, followed by several bioassays, the role of *CaLOX2* in sweet pepper defences has been experimentally validated.

Chapter 4 focuses on the temporal whole-genome transcriptional response of sweet pepper plants in response to western flower thrips herbivory. A high-resolution RNA-Seq approach was used to unravel the temporal and chronological response of several hormonal, and secondary metabolite pathways. I also participated in a similar study to unravel temporal transcriptomic response of *Arabidopsis* during its interaction with WFT (Steenbergen *et al*., 2019). A Bi-directional Blast Homologue (BBH) approach was implemented to investigate commonalities and specifics in sweet pepper and *Arabidopsis* transcriptional responses, both induced upon WFT feeding.

Chapter 5 presents the temporal high-density transcriptional response of white cabbage plants in response to onion thrips herbivory and a comparative analysis on induced defences (transcriptomics) between WFT-induced *Arabidopsis* and sweet pepper and onion-thrips-induced white cabbage.

Finally**, Chapter 6** integrates the key results of this thesis and discusses in the context of the current knowledge of dynamics of plant transcriptional responses to different stresses.

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Chapter 2

Genome-wide identification, classification and expression of lipoxygenase gene family in pepper

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Abstract

Lipoxygenases (LOXs) are non-heme, iron-containing dioxygenases playing a pivotal role in diverse biological processes in plants, including defence and development. Here, we exploited the recent sequencing of the pepper genome to investigate the *LOX* gene family in pepper. Two LOX classes are recognized, the 9- and 13-LOXs that oxygenate lipids at the 9th and 13th carbon atom, respectively. Using two main *in-silico* approaches, we identified a total of eight LOXs in pepper. Phylogenetic analysis classified four LOXs (CaLOX1, CaLOX3, CaLOX4 and CaLOX5) as 9-LOXs and four (CaLOX2, CaLOX6, CaLOX7 and CaLOX8) as 13-LOXs. Furthermore, sequence similarity/identity and subcellular localization analysis strengthen the classification predicted by phylogenetic analysis. Pivotal amino acids together with all domains and motifs are highly conserved in all pepper LOXs. Expression of 13-*LOXs* appeared to be more dynamic compared to 9- *LOXs* both in response to exogenous JA application and to thrips feeding. Bioinformatic and expression analyses predict the putative functions of two 13-LOXs, CaLOX6 and CaLOX7, in the biosynthesis of Green Leaf Volatiles, involved in indirect defence. The data are discussed in the context of LOX families in solanaceous plants and plants of other families.

Key words: Pepper, lipoxygenases (LOXs), phylogenetic analysis, gene transcription, sequence analyses, defence

Introduction

Lipoxygenases (EC 1.13.11.12) are non-heme, iron-containing dioxygenases ubiquitously present in plants, animals and fungi (Brash, 1999). In plants, lipoxygenases (LOXs) are well-known to be involved in several plant processes like tuber development, seed germination, fruit ripening and most importantly in plant defences (Kolomiets *et al.*, 2001; Bailly *et al.*, 2002; Feussner & Wasternack, 2002; Kessler, 2004; Barry & Giovannoni, 2007; Yan *et al.*, 2013). Upon insect or pathogen attack, LOXs oxidize polyunsaturated fatty acids (PUFAs) (linoleic acid, α-linolenic acid and arachidonic acid) constituting a (*Z,Z*)-1,4-pentadiene structural unit and catalyzing it into conjugated hydro-peroxides such as oxylipins (Shibata & Axelrod, 1995; Brash, 1999; Feussner & Wasternack, 2002). Oxylipins such as jasmonates, green leaf volatiles (GLVs) and recently discovered death acids, are known for their roles in defence against herbivorous insects and pathogens (Bell *et al.*, 1995; Allmann *et al.*, 2010; Yan *et al.*, 2013; Shen *et al.*, 2014; Christensen *et al.*, 2015; Losvik *et al.*, 2017). Jasmonates and GLVs are 13-LOX-derived products involved in direct and indirect defences, respectively. In indirect defence, GLVs play a pivotal role in the attraction of natural enemies of the herbivores (ul Hassan *et al.*, 2015). Death acids (10-OPDA, 10-oxo-11-phytodienoic acid, and 10-OPEA, 10-oxo-11-phytoenoic acid) are 9-LOX-derived products that in maize (*Zea mays*) accumulate upon southern leaf blight (*Cochliobolus heterostrophus*) infection resulting in the hampering of growth of fungi and herbivorous insects (Christensen *et al.*, 2015; Christensen *et al.*, 2016).

Plant LOXs are primarily classified into two major classes, 9- and 13-LOXs, based on their positional specificity to oxygenate linoleic acids (LAs) (Feussner & Wasternack, 2002). Moreover, LOXs are also classified as Type-1 and Type-2 based on their primary structure and sequence similarity. LOXs having high sequence similarity (>75%) among themselves and having no plastidic transit peptide are classified as Type-1, whereas LOXs with moderate sequence similarity (>35%) and possessing a plastidic transit peptide are classified as Type-2 (Brash, 1999; Feussner & Wasternack, 2002). All Type-2 LOXs known at present are 13-LOXs, whereas Type-1 LOXs include both 9- and 13-LOXs (Feussner & Wasternack, 2002).

Information on LOXs from several plants has been reported. The *Arabidopsis* genome comprises a total of six *LOXs* (*AtLOX1* - *AtLOX6*) (Umate, 2011). *AtLOX1* is up-regulated in leaves upon pathogen attack and stress-related hormones (Melan *et al.*, 1993); *AtLOX2* is involved in jasmonic acid (JA) biosynthesis (Bell *et al.*, 1995); *AtLOX3* and *AtLOX4* are essential for flower growth and male fertility (Caldelari *et al.*, 2011); *AtLOX5* is important for lateral root development and defence responses (Vellosillo *et al.*, 2007) and *AtLOX6* is expressed in roots and involved in JA synthesis

(Grebner *et al.*, 2013). Among solanaceous plants, different numbers of LOXs are reported in tomato, potato and tobacco. In tomato, *SlLOXA* (*TomLOXA*) and *SlLOXB* (*TomLOXB*) are induced during fruit ripening (Ferrie *et al.*, 1994; Griffiths *et al.*, 1999); *SlLOXC* (*TomLOXC*) participates in production of flavour compounds resulting from fatty acids (Chen *et al.*, 2004); *SlLOXD* (*TomLOXD*) is involved in wound-induced JA biosynthesis, enhancing resistance against herbivores and pathogens (Yan *et al.*, 2013); *SlLOXE* (*TomLOXE*) is expressed in breaker fruit (Chen *et al.*, 2004) and *Sl-LOXF* (*TomLOXF*) enhances systemic resistance stimulated by *Pseudomonas putida* BTP1(Mariutto *et al.*, 2011). In tobacco, *NaLOX1* codes for a 9-LOX and is specifically expressed in roots (Allmann *et al.*, 2010); *NaLOX2* is involved in biosynthesis of GLVs (Allmann *et al.*, 2010; VanDoorn *et al.*, 2010); and *NaLOX3* is involved in JA biosynthesis (Halitschke & Baldwin, 2003; Kessler, 2004). Furthermore, in potato, *StLOXH1* mediates the biosynthesis of volatile C6-aldehydes (GLVs) involved in defence (Leon *et al.*, 2002) and *StLOXH3* is involved in the JA biosynthetic pathway (Royo *et al.*, 1996). Knowledge on LOXs has also been presented in grapevine (Podolyan *et al.*, 2010), kiwifruit (Zhang *et al.*, 2006), rice (Umate, 2011), apple (Vogt *et al.*, 2013), soybean (Shin *et al.*, 2008), cucumber (Liu *et al.*, 2011), and olive (Padilla *et al.*, 2009, 2012).

Pepper (*Capsicum annuum*) is an economically important crop worldwide. It is used e.g. as food, spice, and in pharmacology. There are many biotic and abiotic factors constraining pepper production (Shipp *et al.*, 1998; Pakdeevaraporn *et al.*, 2005; Kulkarni & Phalke, 2009; Kurunc *et al.*, 2011). Despite increasing commercial significance of pepper, the molecular mechanisms underlying different plant processes are still unknown. For instance, to develop resistance against pathogens and insects, identifying genes involved in different defence mechanisms in pepper is important.

To date, no comprehensive knowledge on the pepper *LOX* gene family is available. One 9-LOX, *CaLOX1*, involved in defence and cell-death responses against pathogens has been reported (Hwang & Hwang, 2010). Recently, a second member of the *LOX* gene family (*CaLOX2; Capana03g000103*) was identified, playing a role in JA-regulated defence against Western flower thrips (*Frankliniella occidentalis*) (Sarde *et al.*, 2018). Therefore, there is a need of a genome-wide survey of the *LOX* gene family of pepper. Here, we performed comparative genomics and domain-scan analyses for identification and classification of the *LOX* gene family in pepper. To investigate the conservation levels of pepper LOXs compared to known LOXs of other plant species, we subjected pepper LOXs to sequence analysis, phylogenetic analysis and homology modelling. Furthermore, to investigate the role of pepper *LOXs* in defence mechanisms, we examined their expression upon two treatments: exogenous JA application and exposure to feeding by a natural inducer of JA, the cell-content feeding insect Western flower thrips (WFT). WFT was selected because it is a major pest on

pepper and well-known to induce JA signaling (Hickman *et al.*, 2017; Steenbergen *et al.*, 2018). The resulting data provide insights into putative functions of these genes in pepper.

Materials and Methods

Sequence acquisition and identification of pepper LOXs

Protein sequences of tomato (*Solanum lycopersicum*) lipoxygenases were obtained from the Ensembl Plants database (http://www.ensembl.org) (Yates *et al.*, 2016). LOX sequences from *Brassica oleracea*, *Brassica napus*, *Brassica rapa*, *Arabidopsis thaliana, Nicotiana attenuata*, *Nicotiana tabacum*, *Solanum tuberosum*, *Zea mays* and *Actindia deliciosa,* were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/). *Oryza sativa* and *Cucumis melo* LOX sequences were retrieved from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) and the Melonomics database (http://melonomics.net/), respectively. Two main approaches were used for the identification of the pepper *LOX* gene family. First, BLAST searches were performed locally on the *Capsicum annuum L. Zunla-1* proteome (Qin *et al.*, 2014) using Tomato LOX proteins as queries. Second, the *Capsicum annuum L. Zunla-1* proteome was entirely analyzed for the presence of lipoxygenase gene family signature domains, LOX and PLAT/LH2 (polycystin-1, lipoxygenase, α-toxin domain or lipoxygenase homology) using the Pfam database (v27.0) in the CLC Bioworkbench (https://www.qiagenbioinformatics.com/).

Sequence alignment of lipoxygenases

Alignment of LOX protein sequences was performed using the MUSCLE tool (Edgar, 2004) with default settings. Editing and visualization of alignment was produced in GENEDOC (Nicholas *et al.*, 1997). Sequence logos of conserved regions in pepper LOX proteins were generated by Weblogo 3.3 (Crooks *et al.*, 2004).

Phylogenetic analysis of plant LOXs

Seventy-two plant LOX protein sequences were analyzed, including one known pepper LOX, CaLOX1(L) (*L* stands for 'literature') (Hwang & Hwang, 2010) and eight pepper LOXs identified in the present study. A Maximum likelihood tree using WAG-model (Hall (2013), with 1000 bootstrap replicates was generated using MEGA 7.0 (Kumar *et al.*, 2016). The tree was edited with the Figtree tool (http://tree.bio.ed.ac.uk/software/figtree/).

Sequence analysis and identification of conserved sequences

Conserved sequences and pivotal amino acids were identified by manual observations on pepper LOX alignments in GENEDOC (Nicholas *et al.*, 1997). Molecular weight and isoelectric point of pepper LOX proteins were calculated by protein isoelectric point calculator (Kozlowski, 2016). Subcellular localization analysis was performed using TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/).

Homology modeling of CaLOX1 and CaLOX2 protein

We generated a protein structural model of CaLOX1 and CaLOX2 using the I-TASSER (Roy *et al.*, 2010) database and the resulting model was visualized with YASARA (Krieger *et al.*, 2002).

Plant growth conditions, thrips rearing and bioassays

Sweet pepper [*Capsicum annuum* (Mandy variety, Rijk Zwaan (De Lier, The Netherlands))] plants were grown in a greenhouse at 23-25°C, 70±10 % relative humidity and 16L:8D photoperiod. Four-week-old plants were used in the experiments for both treatments. Western flower thrips (WFT; *Frankliniella occidentalis*) were reared on bean pods (*Phaseolus vulgaris*) in a climate-controlled cabinet (25±2 °C, 70±10 % relative humidity, L16:8D photoperiod). For thrips treatment in the gene expression experiment, five 2nd instar thrips larvae were placed in clip cages and used for infestation on one of the first two true leaves. Empty clip cages were used on control plants for each time point. Samples were harvested at 0, 2, 4, 6, 8, 10 and 24 hours post infestation, frozen in liquid nitrogen and stored at -80°C.

RNA extraction and qRT-PCR

Transcriptional responses of pepper *LOXs* in response to JA treatment (100 µM) and thrips feeding were assessed by qRT-PCR. For JA-treatment, plants were dipped in 100 µM of JA (treatment) or mock-treated with water (control), both mixed with 0.1% of Tween20. One of the first two true leaf samples were harvested at 0, 0.5, 1, 2, 3, 6, 8, 10 and 24 hours post JA application, frozen in liquid nitrogen and stored at -80°C. For both treatments (JA and thrips), control samples were harvested at each time point to rule out the effect of circadian rhythm on the expression of *LOX* genes. Four to five biological replicates (individual plants) were harvested and analysed for each time point and treatment. Each biological replicate comprises one individual plant. Bioline kit (ISOLATE II RNA Plant Kit), in accordance to its protocol, was used for RNA extraction. cDNA synthesis was executed with 1 µg of total RNA with Bio-Rad iScript cDNA synthesis kit. For qPCR, a reaction mixture comprising of 12.5 µl of SYBR Green (Bioline), 1 µl (10µM) of forward and reverse primers, 5.5 µl RNase free-water and 5 µl cDNA was used. The data normalization was performed with a reference gene, *CaActin*. The PCR cycle conditions used were 95°C for 3 mins, fol-

lowed by 40 cycles of 95°C for 15 s, and 60°C for 45 s. Melt curves for each gene were recorded at the end of each cycle. All primers used for qPCR are presented in Supplementary file S1.

Relative gene expression was studied using the geometric mean of Ct (threshold cycles) values (Vandesompele *et al*., 2002) from the reference gene *CaActin* using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Statistical analysis

The gene expression data were subjected to a Student's *t*-test.

Results and Discussion

Identification of lipoxygenase gene family in pepper

A genome-wide search for lipoxygenase genes in pepper was performed by implementing two main approaches: homology search and scanning of the pepper proteome for the presence of "lipoxygenase" and "PLAT/LH2" domains. Both approaches resulted in the identification of eight lipoxygenases in the *Capsicum annuum L. Zunla-1* proteome (Table 1). Several proteins depicting the presence of either one lipoxygenase domain or the PLAT/LH2 domain were excluded from analysis based on arguments of Chen *et al.* (2015).

The total number of LOXs in pepper (8) is similar to that in tomato (7). This number is also close to the number in *Arabidopsis* (6) (Umate, 2011) and kiwifruit (6) (Zhang *et al*., 2006), double the number in olive (4) (Padilla *et al*., 2009, 2012) and much lower than in melon (18) (Zhang *et al*., 2014), cucumber (23) (Liu *et al*., 2011) and grapevine (18) (Podolyan *et al*., 2010). This diverse number of LOXs in different plant species indicates that this gene family has not been conserved during evolution, despite similarities in biochemical functions of the gene family in different plant species (Feussner & Wasternack, 2002).

Genomic and proteomic features of the pepper *LOX* gene family do not differ much (Table 1). At the genomic level, the number of introns varies between 7 and 9, whereas, ORF (Open Reading Frame) length ranges from a minimum of 2379 bp to a maximum of 2748 bp. Most of the pepper *LOXs* are located on Chromosomes 1 and 3, with the exception of *CaLOX8* (Capana11g000928) on Chromosome 11. At the protein level, LOX length varied between 792 and 915 aa, the predicted isoelectric point (PI) ranged between 5.4 and 7.5 and the predicted molecular weight of the proteins ranged from 89959 to 104131 Da. Sequence comparison among pepper LOXs at the protein level shows high sequence identity (33 – 70%) and similarity (48 - 77 %) (Table 2). Taken together, these genomic and proteomic features show a close relation among the pepper *LOXs*, indicative of a gene family.

Phylogenetic analysis of lipoxygenases

To determine the evolutionary relationship and predict the classification of pepper LOXs, a maximum-likelihood phylogenetic tree with 1000 bootstraps was generated. For this, we used sixty-four previously known plus eight pepper LOX protein sequences from twelve different plant species, comprising monocots and dicots. The tree explicitly categorizes plant LOXs into 9-LOXs, 13-LOXs and uncharacterized LOXs. From the identified eight pepper LOXs, four LOXs (CaLOX1, CaLOX3, CaLOX4 and CaLOX5) are characterized, including the previously described CaLOX1(L) (Hwang & Hwang, 2010) into the 9-LOX group and four other LOXs (CaLOX2, CaLOX6, CaLOX7 and CaLOX8) into the 13-LOX group (Fig. 1). Moreover, upon closer examination of the 9- and 13-LOXs major clades, explicit sub-clades of monocot and dicot LOXs are formed indicating that this gene family has evolved differently in monocots and dicots (Fig. 1).

In the 13-LOX clade, pepper LOXs group with well-characterized Solanaceae 13-LOXs like SlLOXD, StLOXH3, NaLOX3, SlLOXF, NaLOX2, StLOXH1 and SlLOXC (Fig. 1). These clusters or sub-clusters among known LOXs and newly identified LOXs may be useful to predict biochemical features and molecular functions of the newly identified pepper LOXs. CaLOX2 clusters with SlLOXD, StLOXH3 and NaLOX3, well-known to be involved in JA biosynthesis (Royo *et al*., 1996; Halitschke & Baldwin, 2003; Kessler, 2004; Yan *et al*., 2013) suggesting that CaLOX2 has a similar function. This matches with our recent study experimentally confirming that *CaLOX2* is involved in JA biosynthesis upon thrips feeding (Sarde *et al*., 2018). Virus-induced gene silencing of *CaLOX2* led to disruption of the jasmonate pathway resulting in enhanced performance of thrips. CaLOX6 clusters with SlLOXF, known to be involved in systemic resistance to *Pseudomonas putida* BTP1 (Mariutto *et al*., 2011). CaLOX7 groups with NaLOX2, StLOXH1 and SlLOXC. These three LOXs are involved in the biosynthesis of green leaf volatiles (Leon *et al*., 2002; Chen *et al*., 2004; Allmann *et al*., 2010; VanDoorn *et al*., 2010). CaLOX8 seems to be related to AtLOX6, known to provide resistance against biotic and abiotic stresses through oxylipin biosynthesis in roots (Grebner *et al*., 2013).

Table 1. Characteristics of lipoxygenases in pepper

Name	Gene IDs	Location coordinates	Nucleotide			Protein			Predicted LOX class	References
			Chr.	No. of Introns	ORF length(bp)	Length (a.a)	Mol. Wt.(Dal)	PI		
CaLOX1	Capana01g003855	261291828:261298825	1	8	2502	833	94198	5.40	9	------
CaLOX2	Capana03g000103	1425452:1429795	3	7	2733	910	102696	6.33	13	(Sarde *et al*., 2018)
CaLOX3	Capana03g003732	235908782:235912945	3	8	2574	857	97476	5.83	9	------
CaLOX4	Capana03g003733	235924316:235929152	3	8	2637	878	99969	5.56	9	------
CaLOX5	Capana01g000180	2592639:2595818	1	8	2463	820	92135	5.78	9	------
CaLOX6	Capana01g001574	52477015:52494659	1	8	2379	792	89959	5.70	13	------
CaLOX7	Capana01g001578	52720202:52733587	1	9	2664	887	99998	5.62	13	------
CaLOX8	Capana11g000928	64997805:65002262	11	8	2748	915	104131	7.54	13	------

Table 2. Pepper LOX protein identities and similarities (%). High, intermediate and low similarity/identity of genes is shown in green, yellow and red color, respectively.

Gene ids	CaLOX5	CaLOX1	CaLOX3	CaLOX4	CaLOX7	CaLOX6	CaLOX8	CaLOX2
CaLOX5		57	52	53	36	33	38	38
CaLOX1	72		59	64	39	35	38	40
CaLOX3	70	73		70	39	36	37	38
CaLOX4	69	77	83		38	35	38	39
CaLOX7	51	56	55	56		70	45	45
CaLOX6	48	50	50	48	77		42	40
CaLOX8	53	54	54	55	65	59		52
CaLOX2	54	57	57	56	62	56	69	

Sequence identity

Sequence similarity

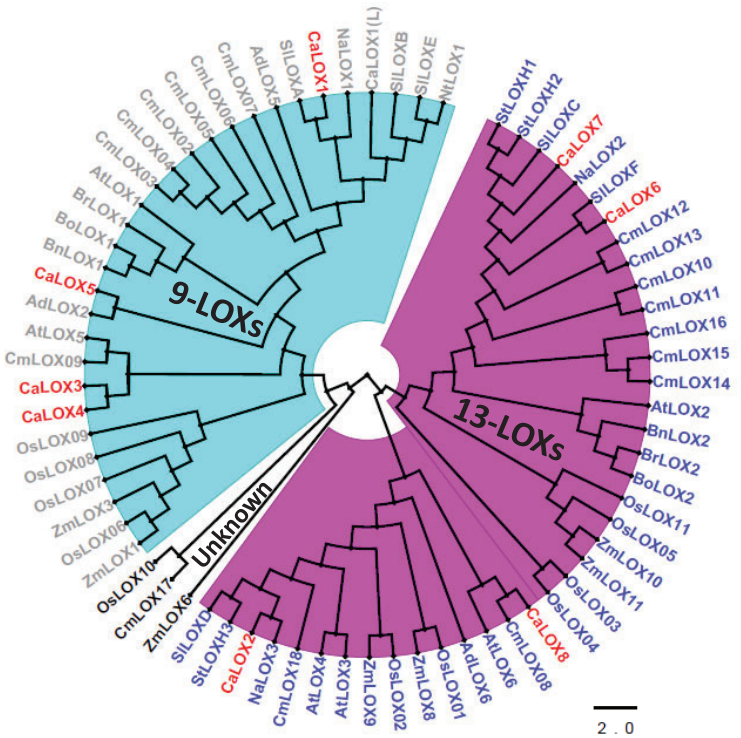

Figure 1: Phylogenetic analysis of plant lipoxygenases. The evolutionary relationship between pepper and other LOX proteins. The tree was generated by MEGA 7 using Maximum Likelihood method with 1000 bootstraps and viewed in Figtree. The scale bar represents the branch length. Different classes of LOXs are depicted in different colors, 13-LOXs in purple; 9-LOXs in blue; unclassified without color. Identified pepper LOXs are highlighted in red color. Species abbreviations used for phylogeny are as follows. At: *Arabidopsis thaliana*, Bo: *Brassica oleracae*, Bn: *Brassica napus*, Br: *Brassica rapa*, Sl: *Solanum lycopersicum*, St: *Solanum tuberosum*, Na: *Nicotiana attenuata*, Nt: *Nicotiana tabacum*, Ca: *Capsicum annuum*, Os: *Oryza sativa*, Zm: *Zea Mays*, Cm: *Cucumis melo*, Ad: *Actindia deliciosa*.

Also in the 9-LOX clade, pepper LOXs cluster with functionally characterized LOXs of other plant species such as AtLOX5, AdLOX2, SlLOXA, SlLOXB (Fig. 1). AdLOX2, that mediates the generation of C6 aldehydes in kiwifruit (Zhang *et al*., 2009), clusters with CaLOX5, suggesting that CaLOX5 has a similar function. CaLOX3 and CaLOX4 form a major clade with AtLOX5 and CmLOX09. AtLOX5 is involved in lateral root development and defence responses (Vellosillo *et al*., 2007). Additionally, relatedness of CaLOX3 and CaLOX4 to each other, suggests that they may be isoforms mediating the same biological process. Furthermore, clustering together of identified CaLOX1 from Capsicum annuum Zunla-1 proteome (Qin *et al*., 2014) and known CaLOX-1(L) reflects their similarity/relatedness, suggesting them to be the same protein. Hwang and Hwang (2010) identified *CaLOX1(L)* independently from cDNA clones and reported it to be involved in defence and cell-death responses against pathogens. Furthermore, CaLOX1 identified here and the previously reported CaLOX1(L) (Hwang & Hwang, 2010) cluster with LOXs like *SlLOXA* and *SlLOXB, two LOXs* that are up-regulated in ripening tomato fruits (Ferrie et al., 1994; Griffiths et al., 1999). Nevertheless, taken together, the predicted functions of pepper LOXs require further experimental validation to characterize their molecular functions, as reported for CaLOX1(L) and CaLOX2 (Hwang & Hwang, 2010; Sarde *et al*., 2018).
Finally, the reported uncharacterized LOXs like OsLOX10, CmLOX17 and ZmLOX6 clearly form an outgroup from the 9- and 13-LOXs (Liu *et al*., 2011; Zhang *et al*., 2014; Cao *et al*., 2016).

Sequence analysis consolidates phylogenetic classification of pepper LOXs

The lipoxygenase family of pepper (CaLOX1 – CaLOX8) is highly conserved with variable sequence identities and similarities with each other (Table 2). It is known that, based on degree of sequence similarity and presence/absence of chloroplast-transit peptide, LOXs are classified into Type-1 or Type-2 (Feussner & Wasternack, 2002; Porta & Rocha-Sosa, 2002). Type-1 LOXs show high sequence similarity (>75%) in the absence of a chloroplast-transit peptide; in contrast, Type-2 LOXs show low sequence similarity and the presence of a chloroplast-transit peptide. The pepper LOXs CaLOX1 and CaLOX3, CaLOX4 and CaLOX5, exhibit high sequence similarity (>70%) and identity (>52%) with each other compared to other LOXs. In contrast, CaLOX2 and CaLOX6, CaLOX7 and CaLOX8 show low sequence similarity among themselves with the exception of CaLOX6 and CaLOX7. CaLOX6 and CaLOX7 show high sequence similarity and identity among themselves, but not when compared to the rest of the LOXs, suggesting that CaLOX6 and CaLOX7 are isoforms of each other. Furthermore, the presence of a chloroplast-transit peptide in sequences of CaLOX2 and CaLOX6, CaLOX7 and CaLOX8 suggests that they are localized in the chloroplast. Collectively, sequence similarity and sub-cellular localization analysis indicates classification of CaLOX1, CaLOX3, CaLOX4 and CaLOX5 into Type-1 and CaLOX2,

CaLOX6, CaLOX7 and CaLOX8 into Type-2.

Furthermore, plant LOXs are also classified into 9- and 13-LOXs, based on their positional specificity of action on the substrate (Feussner & Wasternack, 2002). The presence of Phe608/His608 or Val608 residue predicts LOX activity as 13- or 9-LOX, respectively. Multiple sequence alignment of all pepper LOXs clearly shows the occurrence of valine in CaLOX1 and CaLOX3, CaLOX4 and CaLOX5 classifying them as 9-LOXs and phenylalanine in CaLOX2 and CaLOX6, CaLOX7 and CaLOX8 classifying them as 13-LOXs (Fig. S1). This agrees with the observation that all Type-2 LOXs identified so far are 13-LOXs (Feussner & Wasternack, 2002).

Therefore, both classification methods provide consensus on distribution of pepper LOXs into different classes, thus consolidating our methodology and predictions. Moreover, it also suggests to use the parameters from both approaches in the future for classification of plant lipoxygenases.

High conservation of motifs and pivotal amino acids

Lipoxygenases are characterized by the presence of a 38-residue representative sequence, a substrate-binding domain, an oxygen binding domain and a C-terminal motif (Padilla *et al.*, 2009, 2012). The highly representative 38-residue sequence in lipoxygenases is important for stability of lipoxygenases. In addition, the enzymatic activity or efficiency of lipoxygenases can be severely affected if any of the residues of this sequence is substituted (Chen *et al.*, 2015). This sequence is highly conserved in all the predicted pepper LOXs (Fig. 2A, 2E & 2F). Also, the other motifs like substrate binding, oxygen binding and the C-terminal are conserved among all pepper LOXs (Fig. 2A-F).

Among the conserved amino acids, the three histidine residues (including two from the representative 38-residue sequence) His499, His504, His690 with Asn694 and Ile839 are identified to be vital for binding to non-heme iron (Steczko *et al.*, 1992; Boyington *et al.*, 1997; Feussner & Wasternack, 2002; Porta & Rocha-Sosa, 2002; Padilla *et al.*, 2012).

All these five amino acids appear to be conserved in the pepper LOXs (Fig. S1) with an exception for Ile839 in CaLOX7. Substitution of C-terminal isoleucine with any other amino acid except valine led to inactivation of lipoxygenases, whereas, substitution with valine had positive consequences for enzymatic activity (Chen *et al.*, 1994). Therefore, the absence of a C-terminal motif and the presence of Ile839, essential for non-heme iron binding, leads us to suggest that CaLOX7 may have an altered enzymatic activity.

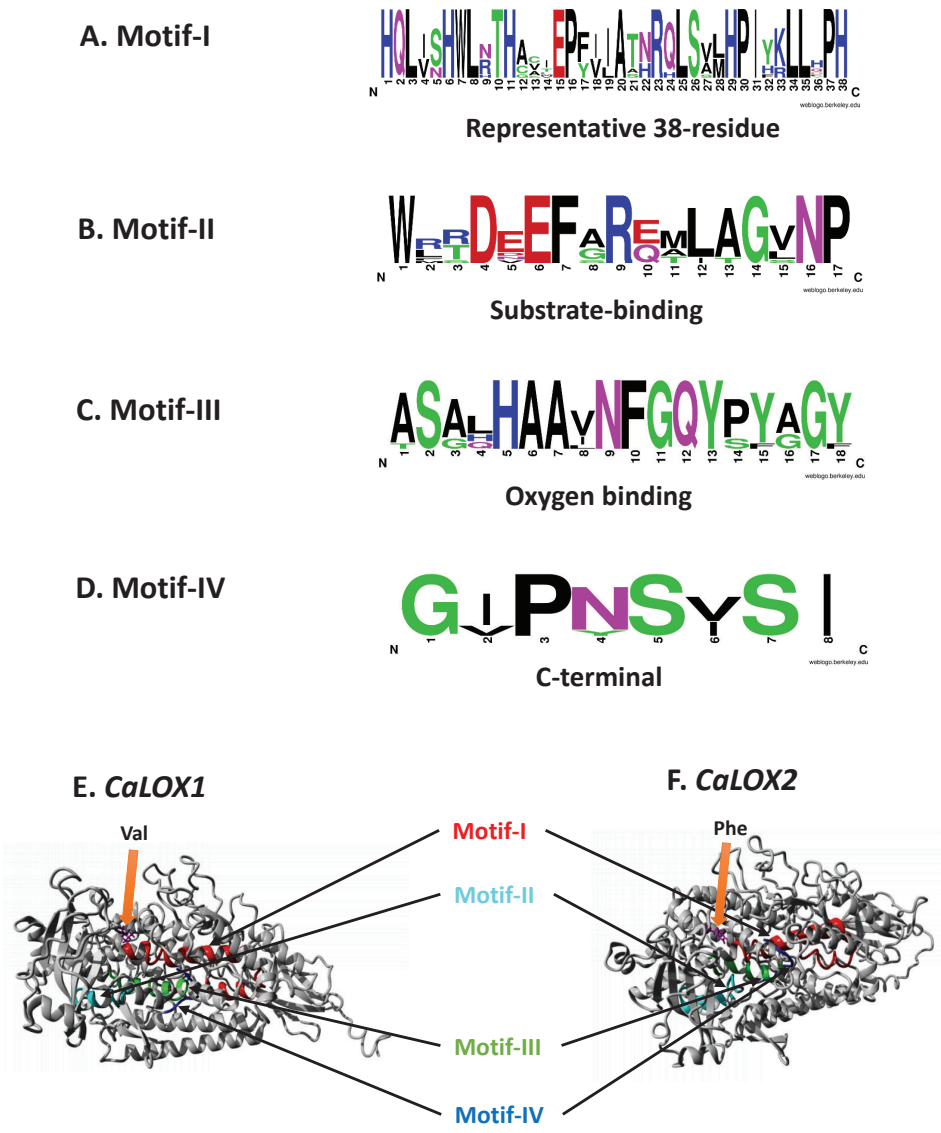

Figure 2. Conservation of sequence motifs in pepper lipoxygenases (A-D) and protein models of CaLOX1 and CaLOX2 (E-F). Highly representative 38-residue motif (A), substrate binding (B), oxygen binding (C) and C-terminal (D) motifs are highly conserved. Protein model of a 9-LOX CaLOX1 (E) and 13-LOX CaLOX2 (F) depicting conservation of highly representative 38-residue (red), substrate binding (cyan), oxygen binding (green) and C-terminal motif (blue) motifs. 13- or 9-LOX activity determinant Phe or Val residue, respectively are depicted.

Moreover, the conserved Val608 or Phe608/His608 residue that are indicative for 9- or 13-LOX activity, respectively (Sloane *et al*., 1991; Hornung *et al*., 1999; Padilla *et al*., 2009, 2012), are found highly conserved in pepper LOXs (Fig. 2E-F and Fig. S1). The determinant residues for inverse substrate orientation and S-stereospecificity of LOXs, Arg and Ala, respectively (Hornung *et al*., 1999; Coffa & Brash, 2004) are well-conserved as well in pepper LOXs (Supplemental Fig. S1). Taken together, the conservation of motifs and pivotal amino acids suggests that functions of pepper LOXs are conserved to their respective homologs in other plant species.

Expression pattern of lipoxygenases upon JA application and thrips feeding

qRT-PCR was performed to investigate the expression dynamics of pepper *LOXs* over time upon thrips feeding or exogenous JA application. Upon thrips feeding, two 13-*LOXs* (*CaLOX2* and *CaLOX7*) are up-regulated for most of the analyzed time points (Fig. 3). Induction of *CaLOX2* occurred after 2h of thrips feeding and remained up-regulated. This gene's involvement in JA biosynthesis has been experimentally supported (Sarde *et al*., 2018). *CaLOX7* is significantly up-regulated at 4h after the start of feeding and remained up-regulated throughout the period suggesting that it may have a role in defence. *CaLOX6* is significantly up-regulated after 10h of feeding. In contrast, all other *LOXs,* i.e. *CaLOX1*, *CaLOX3*, *CaLOX4* and *CaLOX8* did not show induction over time (Fig. 3). *CaLOX4* and *CaLOX8* are significantly down-regulated after 8h of thrips feeding. *CaLOX5* expression is not shown due to its unstable expression resulting in a high degree of variation. This instability of *CaLOX5* expression was also confirmed by its expression pattern in an RNA-seq dataset (Sarde *et al*., unpublished data). In *Arabidopsis*, it is well-known that *LOX* expression is triggered following application of JA due to presence of a positive feedback loop that amplifies JA responses (Hickman *et al*., 2017). Upon exogenous JA application, *CaLOX2,* known to be involved in JA biosynthesis (Sarde *et al*., 2018), shows significant induction after 2h which was maintained until 6h after JA application with exception at 3h (Fig. 4). This instant up- and downregulation of *CaLOX2,* suggests involvement of some feedback mechanism in JA-biosynthetic pathway. *CaLOX6* and *CaLOX7* are also up-regulated upon JA application. Both of them exhibit high expression levels at similar time points i.e., 8h and 24h after JA application. In contrast, the other *LOX* genes, i.e. *CaLOX1*, *CaLOX3*, *CaLOX4* and *CaLOX8,* were not up-regulated at any time point, but exhibited down-regulation at several time points (Fig. 4).

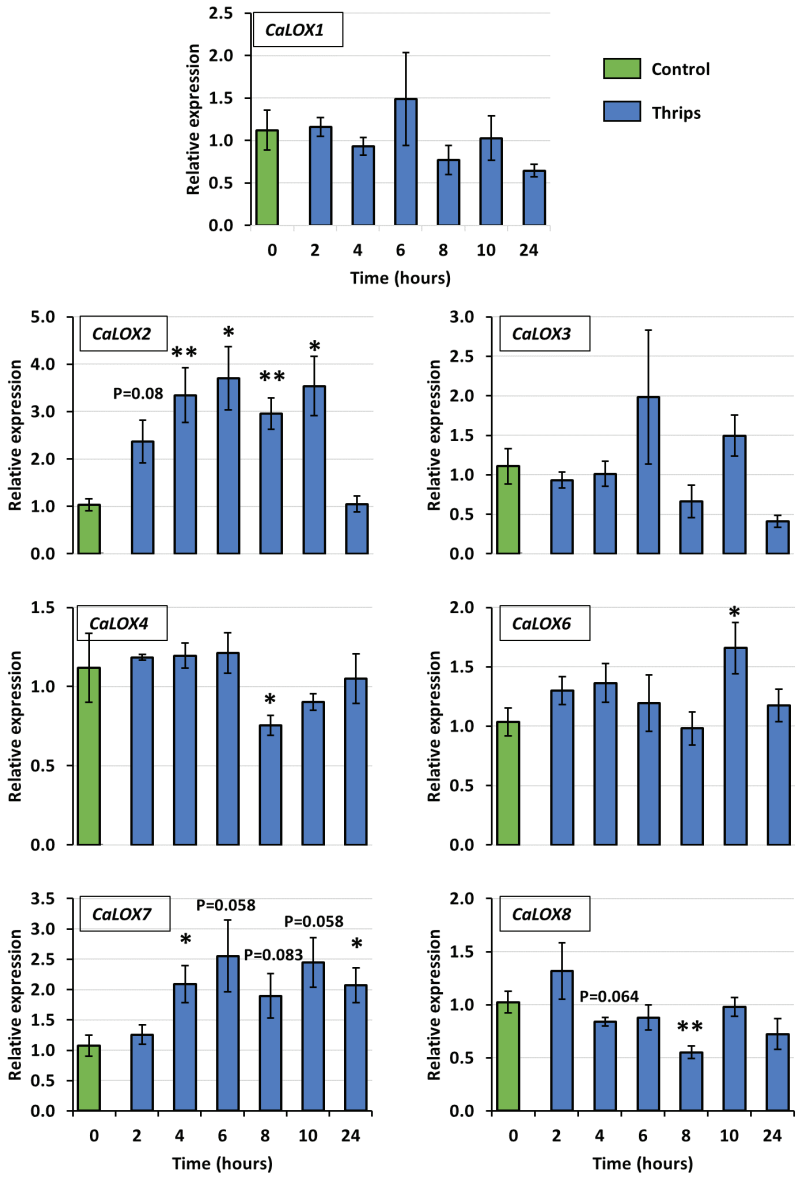

Figure 3. Quantitative RT-PCR (RT-qPCR) of pepper lipoxygenase genes in sweet pepper leaves in response to thrips (*F. occidentalis*) feeding. Five 2nd instar thrips larvae in a clip cage fed on the first true leaf of four-week-old pepper plants. Clip cages without thrips were used on control plants. Expression of the housekeeping gene *CaActin* was used to normalize the expression level of each *LOX* gene at each time point. Relative expression compared to the control for the same time point is presented. Bars represent means ± SE of 4-5 biological replicates. Bars marked with asterisks indicate significant differences (Student's *t*-test) to corresponding control samples for the same time point, * P < 0.05, ** P < 0.01. For bars without asterisk or P value, the P value is > 0.10.

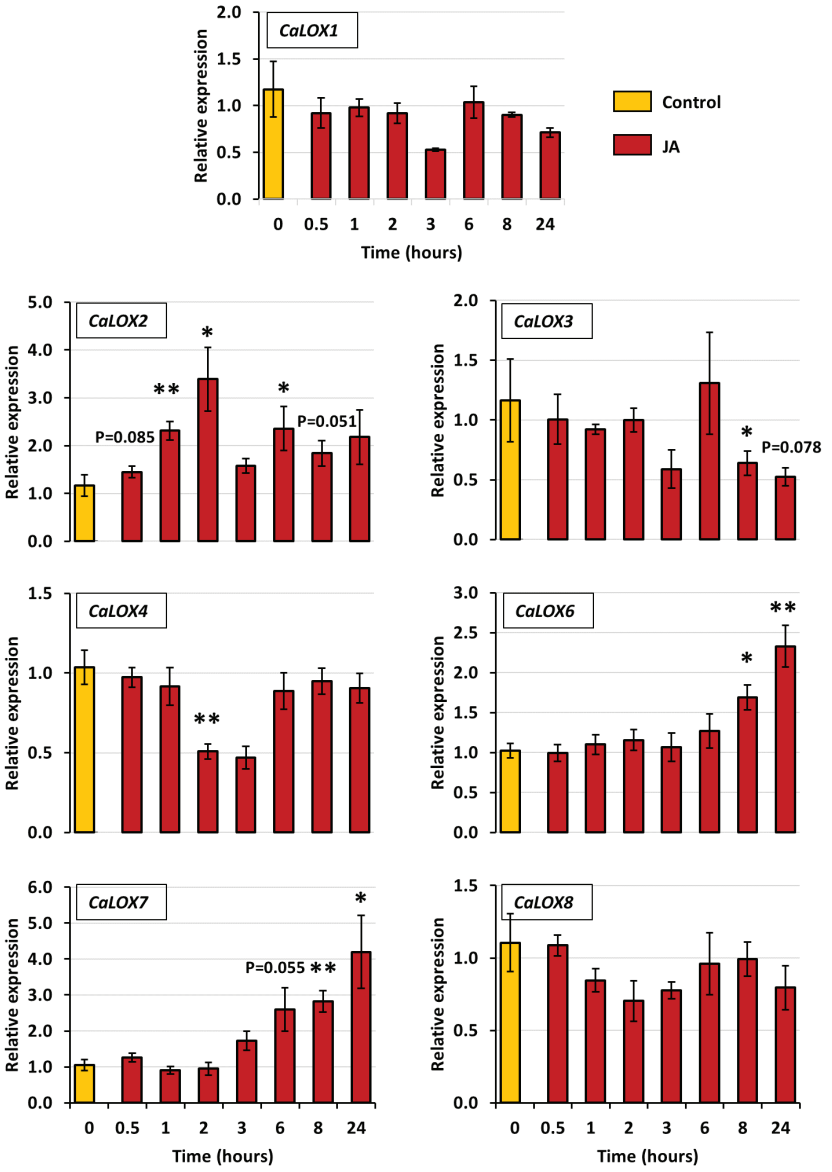

Figure 4. Quantitative RT-PCR (RT-qPCR) of pepper lipoxygenase genes in sweet pepper leaves in response to exogenous JA application. Pepper plants were dipped in water + Tween20 (control) or 100 µM JA + Tween20 (treatment). Expression of the housekeeping gene *CaActin* was used to normalize the expression level of each *LOX* gene at each time point. Relative expression compared to the control for the same time point is presented. Bars represent means ± SE of 4-5 biological replicates. Bars marked with asterisks indicate significant differences (Student's *t*-test) to corresponding control samples for the same time point, * P < 0.05, ** P < 0.01. For bars without asterisk or P value, the P value is > 0.10.

In general, the 9-*LOXs* in pepper (*CaLOX1*, *CaLOX3* and *CaLOX4*) did not show any induction, but rather down-regulation at certain time points in both treatments, i.e. JA application and thrips feeding. This fits to the fact that 9-*LOXs* are especially involved in functions such as plant–pathogen interactions, storage of proteins and tuber development (Feussner & Wasternack, 2002). In contrast, the 13-*LOXs* were more responsive to both treatments, except *CaLOX8*. Similarity of CaLOX7 to NaLOX2 and SILOXC (Fig. 1), both known to be involved in the biosynthesis of green leaf volatiles (GLVs) (Chen *et al.*, 2004; Allmann *et al.*, 2010; VanDoorn *et al.*, 2010; Shen *et al.*, 2014), and it's up-regulation upon both thrips feeding and JA application (Fig. 3 & 4) suggest a role of CaLOX7 in the biosynthesis of GLVs in pepper. Additionally, in tomato *SILOXC*-antisense lines, low expression of both *SILOXC* and *SILOXF* resulted in decreased levels of C5 and C6 leaf volatiles, suggesting a possible synergistic involvement of *SILOXC* and *SILOXF* in the biosynthesis of these plant volatiles (Shen *et al.*, 2014). Therefore, the similarity of CaLOX6 to SILOXF (Fig. 1) and its induction upon both JA application and thrips feeding makes it a potential candidate to test for its synergistic role with CaLOX7 in volatile biosynthesis (Fig. 3 and 4).

Conclusion

In conclusion, this study has identified and classified eight LOXs in pepper. Phylogenetic analysis classified four LOXs as 9-LOXs (CaLOX1, CaLOX3, CaLOX4 and CaLOX5) and four others as 13-LOXs (CaLOX2, CaLOX6, CaLOX7 and CaLOX8) with predictions of their putative functions. Pepper LOX proteins are highly conserved in all lipoxygenase characteristics. Characterization of *CaLOX2* encoding for a LOX that is involved in JA biosynthesis is confirmed by a recent experimental study through a combination of *in-silico,* transcriptional, behavioural, and chemical analyses plus silencing of *CaLOX2* through Virus-Induced Gene Silencing (Sarde *et al.*, 2018). For the other LOXs their function remains to be elucidated. High expression levels of 13-*LOXs* in pepper with support of *in-silico* analysis predict potential candidate genes (*CaLOX6* and *CaLOX7*) that code for enzymes involved in GLV biosynthesis in pepper. Finally, this comprehensive study provides a pepper *LOX* genes repository to further elucidate their functional roles in respective biological processes.

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Chapter 3

Involvement of sweet pepper *CaLOX2* in jasmonate-dependent induced defence against Western flower thrips

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Abstract

Insect herbivory can seriously hinder plant performance and reduce crop yield. Thrips are minute cell-content-feeding insects that are important vectors of viral plant pathogens, and are serious crop pests. We investigated the role of a sweet pepper (*Capsicum annuum*) lipoxygenase gene, *CaLOX2,* in the defense of pepper plants against Western flower thrips (*Frankliniella occidentalis*). This was done through a combination of *in-silico,* transcriptional, behavioral and chemical analyses. Our data show that *CaLOX2* is involved in jasmonic acid (JA) biosynthesis and mediates plant resistance. Expression of the JA-related marker genes, *CaLOX2* and *CaPIN II*, was induced by thrips feeding. Silencing of *CaLOX2* in pepper plants through virus-induced gene silencing (VIGS) resulted in low levels of *CaLOX2* transcripts, as well as significant reduction in the accumulation of JA, and its derivatives, upon thrips feeding compared to control plants. *CaLOX2*-silenced pepper plants exhibited enhanced susceptibility to thrips. This indicates that *CaLOX2* mediates JA-dependent signalling, resulting in defense against thrips. Furthermore, exogenous application of JA to pepper plants increased plant resistance to thrips, constrained thrips population development and made plants less attractive to thrips. Thus, a multidisciplinary approach shows that an intact lipoxygenase pathway mediates various components of sweet pepper defense against *F. occidentalis.*

Key words: Pepper, *CaLOX2,* defence, phylogeny, synteny, gene expression, VIGS, gene silencing, thrips performance

Introduction

In nature, land plants and insects have coexisted for more than 400 million years. Plants perceive herbivorous insects by the specific pattern of tissue disruption and/or chemical cues originating from insects (Bonaventure, 2012; Heidel-Fischer *et al.*, 2014). Plants have three main signal-transduction pathways, each involving a major plant hormone; i.e., jasmonic acid (JA), salicyclic acid (SA) and ethylene (ET), underlying induced defence against attackers such as herbivorous insects (Pieterse *et al.*, 2012; Stam *et al.*, 2014). JA is well-known to be a key regulator of defense, induced by chewing insects and cell-content feeders like thrips (De Vos *et al.*, 2005; Abe *et al.*, 2008; Abe *et al.*, 2009; Pieterse *et al.*, 2012), whereas SA is known to mediate induced plant defense responses against phloem feeders (Zhu-Salzman *et al.*, 2004; Walling, 2008; Pieterse *et al.*, 2012; Tzin *et al.*, 2015). The three major signaling pathways may exhibit crosstalk. For instance, JA and SA usually act antagonistically, but are also reported to act synergistically, or additively (Pieterse *et al.*, 2012; Thaler *et al.*, 2012).

The Western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is a polyphagous and economically important pest. Thrips insert their stylets into plant tissues and ingest cell contents, resulting in a silvery appearance of the damaged area (Steenbergen *et al.*, 2018). They feed on almost all aboveground organs of pepper plants and are considered the most devastating pest in greenhouses, worldwide. Their feeding affects leaf size and photosynthetic capacity, which eventually reduces plant growth and productivity (Steiner, 1990; Welter *et al.*, 1990; Shipp *et al.*, 1998). Thrips often aggregate in narrow crevices on the plants, such as in the flowers, developing fruits, foliage and buds, making them difficult to control. Moreover, they also cause indirect damage by transmitting tospoviruses, such as *Tomato spotted wilt virus* (TSWV) (Maris *et al.*, 2003). Thus, the development of novel approaches to control thrips damage by using knowledge on the molecular mechanisms of plant responses is important.

Various studies have addressed induced plant defenses against leaf-chewing and phloem feeding herbivores (Walling, 2000; Bonaventure, 2012; Heidel-Fischer *et al.*, 2014; Stam *et al.*, 2014; Zust & Agrawal, 2016), whereas much less is known about plant responses to cell-content feeding thrips. To our knowledge, few studies have reported on the role of JA in regulating induced plant defense responses against thrips feeding. In *Arabidopsis* and Chinese cabbage (*Brassica rapa* subsp*. pekinensis*), JA is involved in defense against thrips (Abe *et al.*, 2008; Abe *et al.*, 2009). In tomato, the JA-signaling mutant, *Defenceless1* (*Def1)*, exhibits enhanced susceptibility to thrips feeding (Li *et al.*, 2002; Escobar-Bravo *et al.*, 2017) and a decline in thrips abundance was observed in tomato upon JA application in field conditions (Thaler *et al.*, 2001).

Likewise, cotton and soybean plants also show increased resistance against thrips upon exogenous JA or MeJA application (Omer *et al.*, 2001; Selig *et al.*, 2016). In *Arabidopsis,* a total of 199 genes were differentially expressed (up and down regulated) upon thrips feeding, among which 138 (69%) were JA-responsive genes (De Vos *et al.*, 2005). However, in pepper plants, little is known about the mechanisms underlying defense against insect herbivores.

Lipoxygenases (LOXs) are enzymes encoded by a multi-gene family functioning in different plant developmental and defense processes (Brash, 1999). They are well-known to oxygenate fatty acids. In plants, the main classes are 9-lipoxygenases and 13-lipoxygenases that oxygenate lipids at the 9th and 13th carbon atom, respectively (Feussner & Wasternack, 2002). In JA biosynthesis, the first oxygenation step of linolenic acid is performed by a 13-lipoxygenase (Brash, 1999; Feussner & Wasternack, 2002). Disruption of this 13-LOX has been shown to suppress the JA pathway in several plant species, resulting in enhanced susceptibility to insect herbivory.

In *Nicotiana attenuata* silencing through antisense expression of *NaLOX3*, involved in JA synthesis, suppressed JA synthesis and enhanced herbivore performance (Halitschke & Baldwin, 2003). Similarly, in tomato, overexpression of *TomLOXD* elevated levels of JA and resistance to a caterpillar species (Yan *et al.*, 2013). The 13-LOXs have also been identified in *Arabidopsis* (Bell *et al.*, 1995), potato (Royo *et al.*, 1996), rice (Zhou *et al.*, 2009) and Asian ginseng (Rahimi *et al.*, 2017). In pepper, a 9-LOX, *CaLOX1,* has been identified and reported to be involved in defense and cell-death responses to pathogens (Hwang & Hwang, 2010). However, to date, no 13-type LOX has been characterized in pepper (*Capsicum annuum*).

The main objective of the present study was to identify and characterize a 13-*LOX* gene specifically involved in JA biosynthesis and subsequently elucidate its role in JA-regulated defense of a non-model plant, sweet pepper, against thrips. We performed *in-silico* analysis to identify the pepper 13-lipoxygenase gene that is potentially involved in JA-biosynthesis (termed *CaLOX2*) and evaluated performance and preference of thrips on plants subjected to exogenous application of JA and silencing of the *CaLOX2* gene by **v**irus-**i**nduced **g**ene **s**ilencing (VIGS). For this, we initially investigated the induction of JA- and SA-regulated genes, upon thrips feeding, and long-term effects of exogenous JA application on thrips population size, preference of thrips between JA-treated and non-treated plants, and plant resistance. Furthermore, the consequences of silencing *CaLOX2* through VIGS for production of down-stream JA-related phytohormones and performance and preference of thrips were studied. Thus, this study assesses the role of *CaLOX2* in jasmonic acid-dependent signaling underlying the defense response to thrips feeding in the non-model plant sweet pepper.

Materials and methods

Plant material and thrips rearing

Sweet pepper (*Capsicum annuum*; Mandy variety) (Rijk Zwaan, De Lier, The Netherlands) plants were grown in pots of 12 cm diameter in a greenhouse at 23-25°C with a 16L:8D photoperiod and 70±10% relative humidity. Four-week-old plants were used in the experiments. One day before the experiments, plants were transferred to a climate chamber with controlled conditions (24 ± 1°C, 70 ± 10% relative humidity, 16L:8D photoperiod and light intensity of 70 µmol photons m^{-2} s^{-1}). Western flower thrips (WFT; *Frankliniella occidentalis*) were reared on bean pods (*Phaseolus vulgaris*) in a climate-controlled cabinet (25 ± 2°C, 70 ± 10% relative humidity, L16:8D photoperiod).

Sequence retrieval, homology search and domain analysis

Tomato (*Solanum lycopersicum*) LOX protein sequences were retrieved from the Ensembl Plants genome browser (http://plants.ensembl.org/index.html). Sequences of LOX proteins from other plant species, i.e. *Solanum tuberosum*, *Nicotiana attenuata, Brassica napus,* and *Arabidopsis thaliana* were downloaded from the NCBI repository. The tomato LOXD (*Solyc03g122340*) protein sequence was used to identify *CaLOX2* in pepper. All LOX proteins were subjected to Pfam (v27.0) domain analysis (Finn *et al*., 2014) using CLC Main Workbench (Version 7.6.4). The pepper genome/proteome subjected to analysis was derived from *Capsicum annuum L. Zunla-1* (Qin *et al*., 2014).

Phylogenetic and synteny analysis

Full-length protein sequences were used for alignment with the MUSCLE tool (https://www.ebi.ac.uk/Tools/msa/muscle/) using the default parameters. The obtained alignment was used for construction of a Maximum Likelihood phylogenetic tree with 1000 bootstrap replicates using MEGA5.1 (Tamura *et al*., 2011). Synteny analysis of solanaceous *LOX* genes, specifically involved in the octadecanoid pathway, was performed using the Ensembl Plants genome browser (Yates *et al*., 2016), Mapviewer from NCBI (https://www.ncbi.nlm.nih.gov/mapview/) and the Gene database from NCBI (https://www.ncbi.nlm.nih.gov/gene).

Plasmid construction

The pTRV (*Tobacco rattle virus*)-based VIGS protocol (Wang *et al*., 2013; Senthil-Kumar & Mysore, 2014) was used to generate *CaLOX2*-silenced (TRV:*CaLOX2*) pepper plants. For this purpose, the unique coding gene fragment of the *Lipoxygenase-2* gene of *Capsicum annuum* (*CaLOX2, Capana03g000103*) was selected and ampli-

fied by PCR. The specificity of the selected sequence was checked via the VIGS tool on the Sol Genomics Network (http://vigs.solgenomics.net/). A gene fragment of 282 bp (Supplementary file 1) was cloned into the TRV2-vector and subsequently transformed into *Agrobacterium tumefaciens* GV3101 strain via electroporation. Presence of the *CaLOX2* fragment in the TRV2-vector was verified by restriction digestion and sequencing.

Agro-infiltration and TRV-mediated silencing assays

Agrobacterium tumefaciens GV3101 strains carrying vectors were grown overnight at 28°C in YEB (Yeast Extract Broth) media with appropriate antibiotics (rifampicin 25 µg/ml, kanamycin 50 µg/ml). *A. tumefaciens* cells were centrifuged and resuspended in induction medium containing rifampicin (25 µg/ml), kanamycin (50 µg/ml) and acetosyringone (50 µg/ml) antibiotics for 3-4 h, and thereafter for 1h in infiltration medium with acetosyringone (150 µg/ml). The composition of induction and infiltration medium is provided in Supplementary file 1. *Agrobacterium tumefaciens* GV3101 cultures carrying pTRV1 and pTRV2:*GUS* or pTRV2:*CaLOX2* or pTRV2:*NaPDS* were mixed at a ratio of 1:1 to a final OD_{600} of 1.0 and syringe-infiltrated into cotyledons of two-week-old pepper seedlings (Wang *et al.*, 2013). Due to high sequence similarity between *NaPDS* and *CaPDS*, we used pTRV2:*NaPDS* construct to monitor the proliferation of silencing in pepper plants. The plants prior and post *Agrobacterium*-infiltration were kept in a greenhouse at 23-25°C, 70 ± 10% relative humidity and 16L:8D photoperiod. Efficiency of *CaLOX2* silencing was validated by RT-qPCR.

No-choice and two-choice feeding bioassays post VIGS

To determine the role of *CaLOX2* in sweet pepper resistance to thrips, the youngest fully-grown true leaves of three-week-old Agrobacterium-infiltrated plants were used for no-choice and two-choice thrips bioassays. A no-choice experiment was conducted by using one detached leaf of pTRV2:*GUS* or pTRV2:*CaLOX2* plants. Each detached leaf was placed in a separate Petri dish (140-mm × 20-mm). The petiole was inserted in a 1% agar solution. Five adult female thrips were placed on each leaf in the Petri dish for 6 or 24-h (with 14 replicates) and thrips feeding-associated damage was recorded at these time points. In addition, infested leaves were sampled for qRT-PCR and phytohormone analyses. In two-choice assays, one leaf each of a TRV2:*CaLOX2* and a TRV2:*GUS* plant were placed adjacent to each other in a Petri dish (with 14 replicates). Five adult female thrips were placed in the center of the Petri dish, equidistant from the leaves, and were allowed to feed on the leaves for two 2-3 d. The area of feeding scars (on both abaxial and adaxial leaf sides) was measured using a light microscope and a transparent grid sheet.

JA treatment and bioassays

To test the effect of the induction of JA signaling on sweet pepper resistance to thrips, four-week-old sweet pepper plants were sprayed with 100 µM JA, or mock-treated with water, both mixed with 0.1% of Tween20 (detailed protocol in Supplementary file 1). The treatment was conducted one day before the bioassay. The experiments (non-choice and choice assays) were performed in a climate chamber under controlled conditions (24 ± 1°C, 70 ± 10% relative humidity, 70 µmol photons m^{-2} s^{-1} light intensity and 16L:8D photoperiod). For a non-choice feeding bioassay, 25 female adult thrips were placed on each JA-treated and non-treated whole pepper plants confined in transparent plastic cages covered with mesh on top. After 14 d, the number of their offspring (first and second-instar larvae) and feeding damage on plants was assessed. The experiment was executed twice, one for assessment of offspring number (with 10 replicate plants) and one for assessment of feeding damage (with 12 replicate plants). In preference (choice) assays (with 11 replicates), a JA-treated and a mock-treated plant were positioned on either side of a transparent plastic box (height: 310 mm, width: 440 mm, length: 710 mm) and 50 adult female thrips were placed halfway between them. The boxes were incubated in a climate chamber with controlled conditions (24 ± 1°C, 70 ± 10% relative humidity, 16L:8D photoperiod). Two d later, the numbers of thrips on each plant were recorded.

RNA isolation and qRT-PCR

To test the effect of thrips feeding on JA and SA-associated defense genes, five second instar thrips larvae (L2) were introduced into a clip cage and allowed to feed on one of the first two true leaves of four-week-old sweet pepper plants. Plants with clip cages without thrips served as controls. At 0, 5, 10 and 24 h after infestation, leaves were sampled for gene expression analyses of JA- and SA-associated marker genes. For each treatment and time point, 4-5 biological replicates were analyzed, each replicate consisting of one individual plant. RNA extraction was executed using the Bioline kit, in accordance to the manufacturer's protocol. cDNA was synthesized from 1 µg of total RNA with iScript cDNA synthesis kit (Bio-Rad). For qPCR analysis, a 25 µl reaction mixture, containing 1 µl (10µM) of forward and reverse primers, 12.5 µl of SYBR Green Supermix (Bio-Rad) and 5 µl cDNA, was used. The reference gene, *CaACTIN*, was used as normalizer for determining the relative expression of JA-related genes (*CaLOX2; Capsicum annuum Lipoxygenase 2* and *CaPIN II; Capsicum annuum Protease Inhibitor II*) and an SA-dependent gene (*CaPR1; Capsicum annuum Pathogenesis Related 1*). The following PCR conditions were used: 3 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 45 s at 60°C. At the end of each qPCR, the melting curve of each gene was recorded. All primers used for qPCR pre and post VIGS are presented in Supplementary file 1. Relative gene expression was analyzed

using the geometric mean of threshold cycle (Ct) values (Vandesompele *et al.*, 2002) for the reference gene *CaACTIN* with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Hormone quantification

Leaf samples (100 mg each) from pTRV2:*GUS* and pTRV2:*CaLOX2* plants were flash-frozen in liquid nitrogen and stored at -80°C. High-performance liquid chromatography–mass spectrometry (HPLC-MS/MS) was used to quantify JA (jasmonic acid), JA-Ile (jasmonic acid isoleucine) and OPDA (12-oxo-phytodienoic acid) content, according to the method described in Trapp *et al.* (2014).

Statistical analysis

Data on gene expression and hormone content were log-transformed prior to statistical analyses. The data on gene expression, thrips feeding, gene silencing efficiency post VIGS and hormonal quantification were all subjected to a Student's *t*-test. Moreover, thrips two-choice preference data were expressed as the proportion of thrips detected on either treatment. The data were analyzed by a t-test within each treatment to determine if the proportion of thrips significantly differed from 0.5, as previously described (Grostal and Dicke (1999). The data of the two-choice feeding experiment post VIGS treatment were analyzed by a paired t-test. These analyses were performed using software IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA). Biological replicates used for statistics each consist of one individual plant.

Results

Identification of *CaLOX2* in pepper

To identify the *LOX2* homolog in pepper, all sequences of tomato LOX proteins were used as queries in blast searches for a genome-wide search against the protein database of *Capsicum annuum L. Zunla-1*. This resulted in the identification of several LOX proteins in pepper (Sarde *et al.*, 2018). These LOX homologs were further scanned for the presence of PLAT/LH2 and LOX domains that are hallmarks of the lipoxygenase gene family (Chen *et al.*, 2015), using the Pfam database. To narrow down the selection towards the specific LOX protein induced upon herbivory or wounding in sweet pepper, the closest homolog of the tomato *LOXD* gene, well-known to have a similar function (Yan *et al.*, 2013), was selected from pepper (*CaLOX2, Capana03g000103*) and further subjected to phylogenetic and synteny analysis.

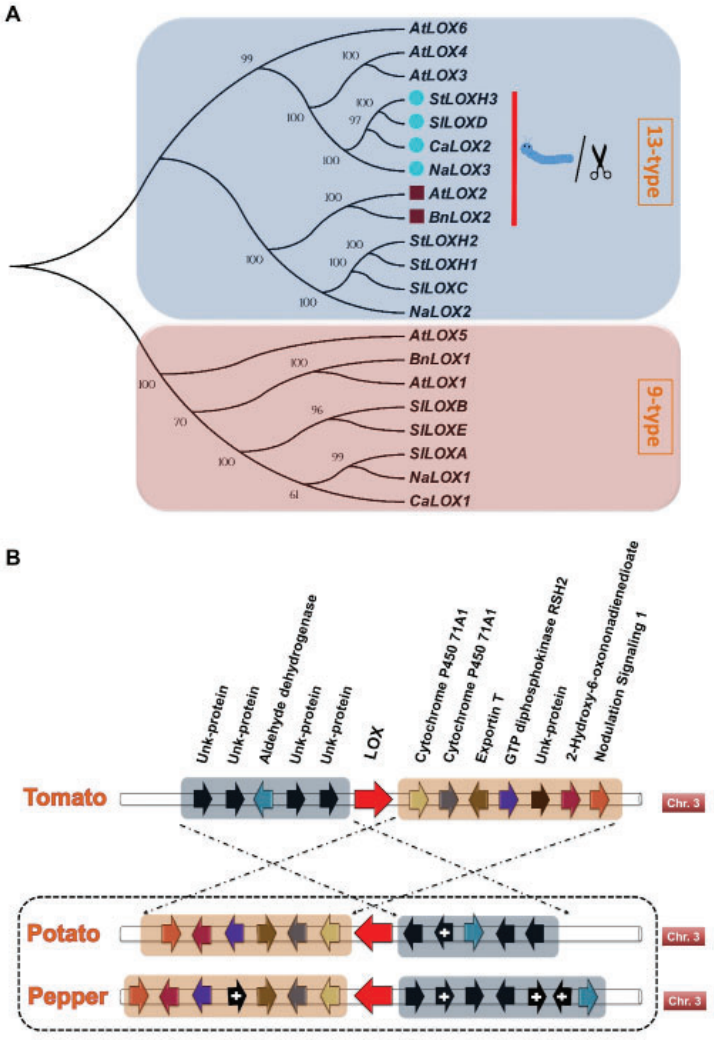

Figure 1. Phylogenetic and synteny analyses of lipoxygenases. (**A**) Phylogenetic relationship of lipoxygenases of six species from the Brassicaceae and Solanaceae families. The tree was generated by MEGA 5.1 using the Maximum Likelihood method with 1000 bootstraps. Accession numbers or Gene IDs of sequences used to construct the phylogenetic tree are as follows. *Arabidopsis thaliana: AtLOX1,* AAA32827*; AtLOX2,* AAA32749*; AtLOX3,* AT1G17420*; AtLOX4,* AT1G72520*; AtLOX5,* AT3G22400*; AtLOX6,* AT1G67560*; Brassica napus: BnLOX1,* AAO03558*, BnLOX2,* NP_001303054*; Solanum lycopersicum: SlLOXC,* AAB65766*; SlLOXD,* AAB65767; *SlLOXB,* AAA53183*; SlLOXE,* AAG21691*; SlLOXA,* AAA53184; *Solanum tuberosum: StLOXH2,* CAA65268*; StLOXH3,* CAA65269*; StLOX1,* AAB67858*; Capsicum annuum: CaLOX2,* Capana03g000103; *CaLOX1,* ACO57136*; Nicotiana attenuata: NaLOX1,* AAP83134*; NaLOX2,* AAP83137*; NaLOX3,* AAP83138. (**B**) Syntenic organization of tomato (*SlLOXD*), potato (*StLOXH3*) and pepper (*CaLOX2*) *LOX* genes. Black arrows with and without "+" sign depict genes that are not identical to synteny of tomato or unknown (uncharacterized) genes, respectively.

Phylogenetic analysis was conducted to analyze the evolutionary relationship between several LOX proteins across different species of the Brassicaceae and Solanaceae families. The phylogenetic tree (Figure 1A) clearly represents two major clades of 13-type and 9-type LOX proteins. In the 13-type clade, the related pepper LOX2 protein is positioned in the sub-clade of LOX proteins within the Solanaceae family that are known to be involved in JA biosynthesis. Similarly, this sub-clade appears closer to the Brassicaceae sub-clade comprising LOX proteins that have a similar function (Figure 1A), suggesting a role of *CaLOX2* in JA biosynthesis.

Furthermore, genomic locations and assessments of syntenic maps offer insights into the conservation of genes across organisms. In the model plant of the Solanaceae family, tomato, the *TomLOXD* gene, well-known to be induced upon herbivory, is flanked by five genes on one side and seven genes on the other side (Figure 1B). The synteny is similar, but in reverse order with some exceptions of additional, uncharacterized or unknown genes for its counterpart *LOX* genes in potato (*LOXH3*) and pepper (*CaLOX2*). Moreover, localization of this gene is still intact on Chromosome 3 in pepper, tomato and potato. Taken together the *in-silico* analysis suggests a possible role of *CaLOX2* in the octadecanoid pathway.

JA and SA-related marker genes are up-regulated upon thrips feeding

We analyzed the expression of JA and SA-related marker genes in sweet pepper in response to thrips feeding in order to determine the role of JA in defense against thrips. Expression of the *in-silico* identified *CaLOX2* gene was induced upon thrips feeding at all three time points sampled (Figure 2A). Similarly, expression of the downstream JA-responsive gene, *CaPIN II,* was also up-regulated upon thrips feeding (Figure 2B). Moreover, upon thrips infestation the SA-responsive gene, *CaPR1*, did not show significant induction after 5h but was up-regulated after 10 and 24h of thrips feeding (Figure 2C).

Exogenous JA application negatively affects thrips feeding and preference

Because thrips feeding induces the transcription of JA-related genes, we first investigated the effect of exogenous JA application on thrips population build-up and its feeding. For this purpose, twenty-five adult females were introduced onto control and JA-treated plants. After two weeks, the larvae and adults were counted on each plant. The JA-treated plants had significantly fewer larvae and adult thrips compared to control plants (Figure 3A, B). Control plants had 2.8 times more larvae (first and second instars; Figure 3A) and 3.5 times more adults (Figure 3B) than JA-treated plants. This indicates that JA underlies pepper defense against thrips.

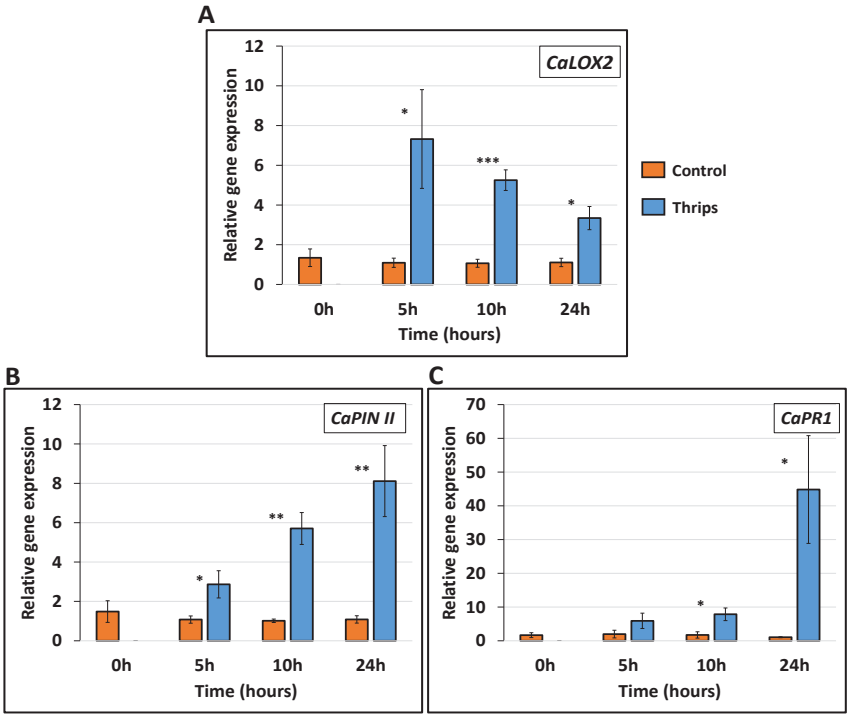

Figure 2. Quantitative RT-PCR (RT-qPCR) of JA and SA-related marker genes in sweet pepper leaves in response to thrips (*F. occidentalis*) feeding. (A) *CaLOX2* and **(B)** *CaPIN II*, as marker genes for the JA-pathway and **(C)** *CaPR1*, as marker gene for the SA-pathway. Five 2nd instar thrips larvae fed locally (confined in clip cages) on the first true leaf of four-week-old pepper plants. Empty clip cages were used on control plants. The expression level of each gene was normalized to the expression of the housekeeping gene *CaACTIN*. Bars represent mean ± *SE* (n = 4-5 biological replicates). Bars marked with asterisks indicate significant differences (Student's t-test), *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001.

We subsequently investigated the effect of exogenous JA application on thrips feeding, in the same experimental setup. Significantly more feeding damage was recorded on control plants (450 mm^2) compared to JA-treated plants (216 mm^2) (Figure 3C). These results further support the involvement of JA in resistance of pepper against thrips.

Finally, we investigated the effect of JA on host plant preference of thrips. To this end, we placed 50 adult females halfway between a control and a JA-treated plant and counted the number of thrips on each plant after 2 days; thrips significantly preferred control plants over JA-treated plants (Figure 3D).

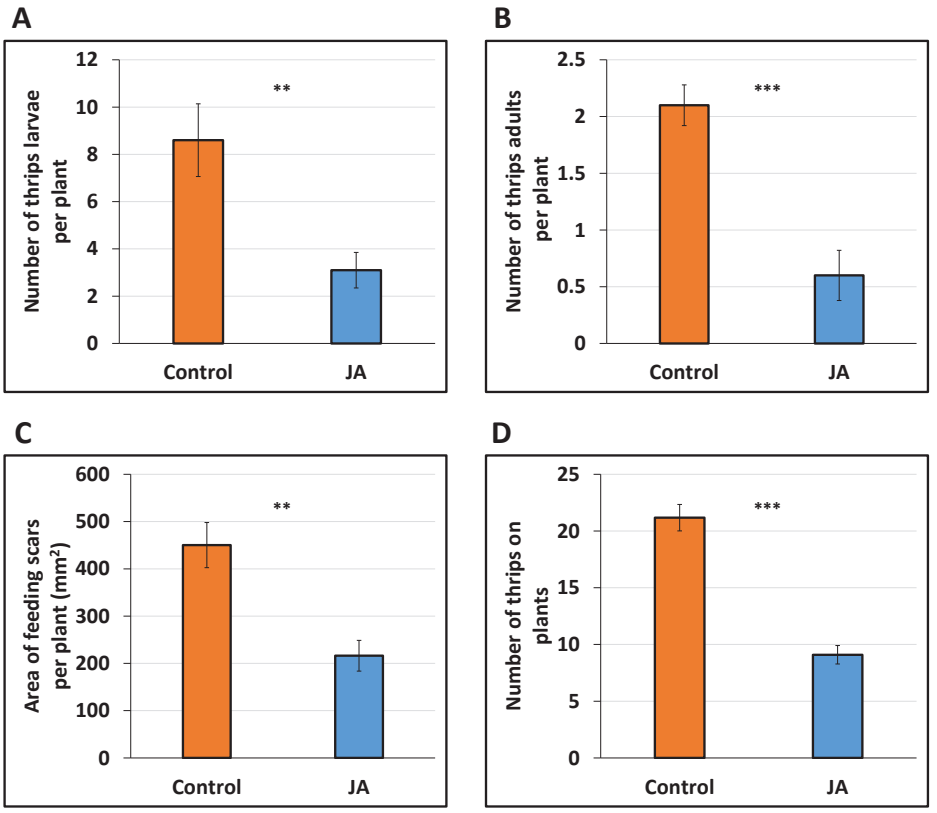

Figure 3. No-choice (thrips population and feeding damage) and choice (preference) tests of Western flower thrips upon exogenous application of JA (100 µM). (A, B) Effect of JA application on thrips population and (C) feeding damage. Twenty-five adult females fed on 4-week-old sweet pepper plants for two weeks. Water + Tween20 (control) or 100 µM JA + Tween20 (treatment) was applied 1 day before thrips were introduced on pepper plants. Number of thrips larvae (A), thrips adults (B) and feeding damage (C) in mm^2 caused by thrips feeding on plants was assessed after two-weeks. Mean ± SE based on ten and twelve biological replicates for thrips population and feeding damage, respectively. Asterisks indicate significant differences (Student's t test), **p-value < 0.01, ***p-value < 0.001. (D) Effect of exogenous JA on host plant preference of thrips (choice experiment). Mean ± SE based on eleven biological replicates. Asterisks indicate significant differences (Student's t test, t= 7.598); ***p-value < 0.001.

VIGS of *CaLOX2* suppresses *CaLOX2* expression

To study the involvement of *CaLOX2* in the JA pathway, we silenced *CaLOX2* in pepper plants using VIGS. The unique region of 282 bp of the *CaLOX2* coding region (Supplementary file 1) was selected, using CLC bio-workbench and its specificity was confirmed using the VIGS tool from the Sol Genomics Network.

To assess the efficiency of VIGS, *CaLOX2* transcript levels were quantified using

qRT-PCR in GUS-vector control (TRV:*GUS*) and *CaLOX2*-silenced (TRV:*CaLOX2*) pepper leaves infested with thrips. *CaLOX2* expression was significantly induced, at both time points, in GUS-vector control leaves (Figure S1). In contrast, in *CaLOX2*-silenced pepper leaves, *CaLOX2* transcript levels were significantly decreased compared to GUS-vector control leaves, both experiencing thrips feeding, indicating that *CaLOX2* silencing was effective in suppressing *CaLOX2* induction (Figure 4A).

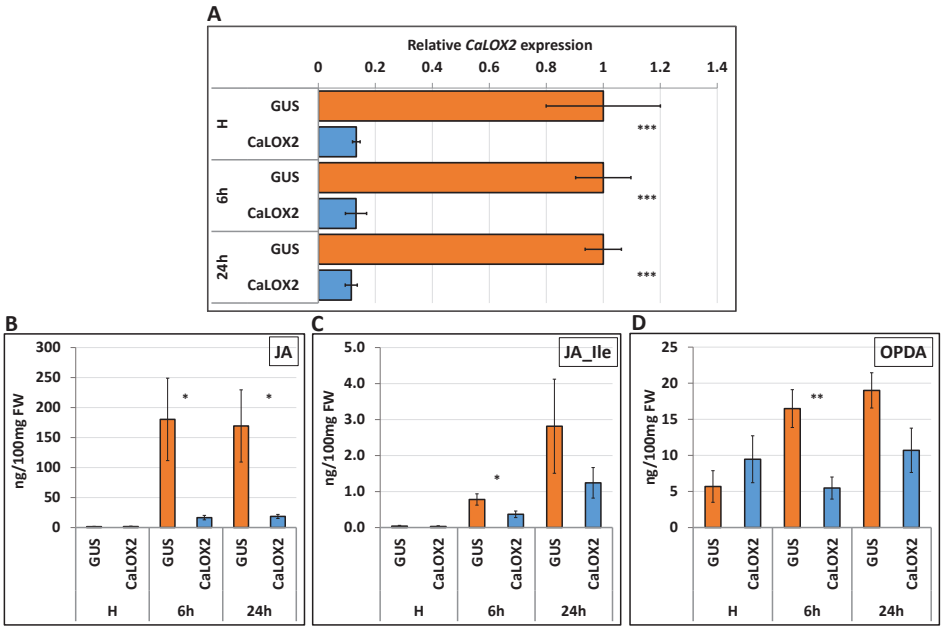

Figure 4. Quantitative RT-PCR (RT-qPCR) of *CaLOX2* and phytohormone quantification in GUS-vector control and *CaLOX2*-silenced leaves infested with western flower thrips for 6 h and 24 h. **(A)** Silencing efficiency of *CaLOX2*. H, healthy leaves; GUS, β-Glucuronidase; *CaLOX2, C. annuum Lipoxygenase2*. The *C. annuum* actin gene was used for normalization in qPCR. Data are mean ± *SE* of 14 biological replicates from two independent experiments. Asterisks indicate significant differences (Student's t test), ***p-value < 0.001. **(B, C, D)** Quantification of JA and its derivatives. **OPDA**, 12-oxo-phytodienoic acid**; JA**, Jasmonic acid; **JA-Ile**, Jasmonic acid isoleucine. Five adult females fed on a detached single leaf of a 5-week-old plant. Data are mean ± *SE* of 6 biological replicates. Asterisks indicate significant differences (Student's t test), *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

Silencing of *CaLOX2* compromises jasmonate accumulation

To investigate the effect of *CaLOX2* silencing on the accumulation of JA and its derivatives in response to thrips attack, we assessed the levels of phytohormones in leaves with the GUS-vector and *CaLOX2*-silenced pepper plants infested with thrips (Figure 4). *CaLOX2* silencing resulted in lower levels of OPDA, JA and JA-Ile. The differences were statistically significant at 6 h for all three phytohormones and at 24 h for OPDA and JA. This indicated that *CaLOX2* silencing compromised the induc-

tion of JA, and its derivatives JA-Ile, and OPDA in *CaLOX2*-silenced pepper leaves subjected to thrips feeding (Figure 4B - D). These results support a role of *CaLOX2* in the octadecanoid pathway leading to the biosynthesis of JA and JA-Ile, as induced upon herbivory.

Suppression of *CaLOX2* confers increased susceptibility to thrips feeding

To assess the consequence of *CaLOX2* silencing for thrips feeding and preference, we quantified thrips feeding damage on leaves of GUS-vector control and *CaLOX2*-silenced pepper plants. This was done in no-choice (Figure 5A) and choice (Figure 5B) tests. Injury resulting from thrips attack was significantly greater on *CaLOX2*-silenced plants than on non-silenced (GUS) plants, in a no-choice test, at both time points, indicating that a functional *CaLOX2* reduces the feeding rate of thrips individuals (Figure 5A). Furthermore, when provided with a choice between silenced leaves (*CaLOX2*-silenced) and non-silenced (GUS-vector) leaves, thrips clearly preferred to feed on *CaLOX2*-silenced plants (Figure 5B). Taken together, these results support the role of *CaLOX2* in the octadecanoid pathway of induced defense.

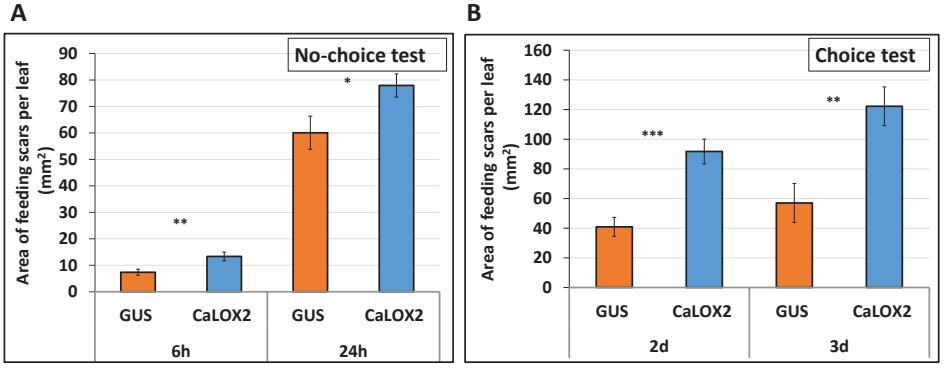

Figure 5. Effect of *CaLOX2* silencing by VIGS in sweet pepper plants on thrips feeding in no-choice and thrips preference in choice tests. (A) Area of feeding scars per leaf after 6 and 24 h of thrips feeding (5 female thrips adults per leaf) in no-choice assay. Mean ± SE, based on fourteen biological replicates from two independent experiments. Asterisks indicate significant differences (Student's t test), *p-value < 0.05, **p-value < 0.01. (B) Area of feeding scars per leaf after 2 and 3 d of infestation by 5 female thrips adults in choice assay. Mean ± SE, based on fourteen biological replicates from two independent experiments. Asterisks indicate significant differences (paired t-test), **p-value < 0.01, ***p-value < 0.001.

Discussion

Through a combination of an *in-silico* analysis and transcriptional, behavioral and chemical analyses, we show that *CaLOX2* (Capana03g000103) is activated by thrips feeding, and involved in the octadecanoid pathway, leading to the phytohormone JA

and its active conjugate JA-Ile, resulting in induced defense against thrips in sweet pepper. Although jasmonates have been reported as important players underlying induced plant defense against various herbivorous insect species (Wasternack, 2007; Howe & Jander, 2008), most research has focused on defenses against leaf-chewing or phloem-feeding herbivores (De Vos *et al.*, 2005; Bonaventure, 2012; Heidel-Fischer *et al.*, 2014; Stam *et al.*, 2014; Zust & Agrawal, 2016).

By contrast, relatively little is known about induced plant defenses in response to cell-content feeders like thrips. Most knowledge on inducible defense against thrips is available for model plants, such as *Arabidopsis thaliana* and tomato, for which specific well-characterized mutants are available (Li *et al.*, 2002; Abe *et al.*, 2008; Abe *et al.*, 2009; Abe *et al.*, 2012). Disentangling the molecular mechanisms underlying induced defense against thrips in crops will be valuable in the selection of resistant varieties.

Few studies have reported that jasmonates regulate induced defenses against thrips (Li *et al.*, 2002; Abe *et al.*, 2008; Abe *et al.*, 2009). Due to the relatively limited availability of genetic tools and genomic information, little is known about the biosynthesis of JA and the role of jasmonates in modulating defense responses in non-model plants, such as pepper. Nonetheless, recent knowledge on genomic information and valuable tools like VIGS provides valuable resources to investigate the role of the octadecanoid pathway in defense against thrips feeding. We employed these tools to identify *CaLOX2* as being the *LOX* gene involved in the JA-pathway and resistance of cultivated pepper to *F. occidentalis*.

By *in-silico* analysis, we identified *CaLOX2* as the gene involved in the octadecanoid pathway. *CaLOX2* is a 13-type LOX that is phylogenetically close to functionally similar orthologues in tomato (*SlLOXD*), potato (*StLOXH3*) and tobacco (*NaLOX3*), which are specifically involved in the biosynthesis of JA (Figure 1A) (Royo *et al.*, 1996; Kessler *et al.*, 2004; Yan *et al.*, 2013). Moreover, chromosomal locations and comparisons of syntenic maps show that flanking genes are conserved, with some exceptions of uncharacterized or additional genes, and that the *CaLOX2* gene is positioned on Chromosome number 3, across selected plants from the Solanaceae family. This supports the evolutionary significance of this gene to the plants and supports a role of *CaLOX2* in the octadecanoid pathway, as further reinforced experimentally in our study.

Our findings clearly show that the JA-related genes, *CaLOX2* and *CaPIN II*, are up-regulated at all time points upon thrips feeding, as also recorded for respective JA-related genes in *Arabidopsis* and Chinese cabbage (*Brassica rapa subsp. pekinensis*) (De Vos *et al.*, 2005; Abe *et al.*, 2008; Abe *et al.*, 2009). Moreover, the up-regulation of *LOX* genes was also recorded in cabbage (*Brassica oleracea L.)* and tomato, upon

feeding by another cell-content feeding herbivore, the spider mite *Tetranychus urticae* (Li *et al*., 2002; Zheng *et al*., 2007). In contrast, no up-regulation of *CaPR1* (SA-related) was recorded until 10 and 24 h after initiation of thrips feeding, similar to what has been recorded for the SA-related genes *PR1* and *BGL.2* in *Arabidopsis* (Abe *et al*., 2008). De Vos *et al.* (2005) reported that SA levels were elevated after 12 h of thrips feeding, corresponding with induction of SA-related genes at later time points. Possibly, thrips manipulate plant defense by inducing SA, to stimulate antagonistic crosstalk with the JA pathway to interfere with plant defense (Abe *et al*., 2012; Stam *et al*., 2014).

Both exogenous application of JA and silencing of *CaLOX2,* which interferes with JA induction demonstrate that JA is involved in inducible defense against thrips. JA is also involved in inducible defense against another cell-content feeding herbivore, the spider mite *Tetranychus urticae* in Lima bean and tomato plants (Dicke *et al*., 1999; Li *et al*., 2002; Gols *et al*., 2003; Ament *et al*., 2004). Similar induction of resistance against thrips, by the application of exogenous JA, was also recorded in *A. thaliana*, *Brassica rapa* and *S. lycopersicum* (Li *et al*., 2002; Abe *et al*., 2008; Abe *et al*., 2009).

There are several homologs of *LOX* genes in a wide range of plants (Zhang *et al*., 2006; Podolyan *et al*., 2010; Liu *et al*., 2011; Umate, 2011; Zhang *et al*., 2014; Chen *et al*., 2015). They have been thoroughly characterized and reported to be involved in several plant biological processes, such as plant defense, tuber growth, germination of seeds, and fruit ripening (Kolomiets *et al*., 2001; Bailly *et al*., 2002; Porta & Rocha-Sosa, 2002; Barry & Giovannoni, 2007; Abe *et al*., 2008).

Usually, there is at least one *LOX* homolog induced upon herbivory and involved in the biosynthesis of JA (Kessler *et al*., 2004; Zhou *et al*., 2009; Allmann *et al*., 2010; Zheng *et al*., 2011; Yan *et al*., 2013). These phenomena have been well studied in model plants, but little is known in non-model plants. In order to study the role of octadecanoid pathway in different biological processes in plants, JA-deficient mutants have been generated. In *Arabidopsis*, the *Coi1*-mutant is often used; this mutant is defective in the JA-Ile receptor (Xie *et al*., 1998). In tomato, several mutant lines, including *Def-1*, *Spr-1*, and *Spr-2*, are available (Howe *et al*., 1996; Howe & Ryan, 1999; Li *et al*., 2002).

Nonetheless, the genomic target region of JA-deficient mutants in tomato is still unclear. In rice and tobacco, a specific homolog of *LOX* was targeted to generate JA-deficient plants through transformation (Kessler *et al*., 2004; Zhou *et al*., 2009). Silencing of *OsHI-LOX* or *Na-LOX3* in rice and tobacco, respectively, interfered with the induction of JA upon herbivore feeding (Halitschke & Baldwin, 2003; Zhou *et al*., 2009; Lu *et al*., 2015). Likewise, in a recent study in barley, overexpression and down-regulation of *LOX2.2* affected the expression of JA-related genes depicting its

role in JA-biosynthesis (Losvik *et al.*, 2017).

The present study used gene silencing of *CaLOX2*, instead of transformation, and demonstrates that *CaLOX2* interferes with the production of not only JA and but also other jasmonates, such as OPDA and JA-Ile, upon thrips feeding on pepper, underlining its role in the octadecanoid pathway. Moreover, our data show that targeting a specific 13-type *LOX* gene in a non-model plant can be effective to suppress the entire jasmonate cascade.

Silencing *CaLOX2* clearly resulted in enhanced feeding and preference of thrips for *CaLOX2*-silenced leaves, compared to control leaves. Furthermore, in *Nicotiana attenuata* and rice, JA-silenced plants were more vulnerable to herbivorous insects (Kessler *et al.*, 2004; Zhou *et al.*, 2009). Moreover, silencing *LOX3* in tobacco influenced herbivore community composition under field conditions (Kessler *et al.*, 2004). Such effects are likely the consequence of altered plant phenotype in terms of secondary metabolites or proteinase inhibitors whose biosynthesis is modulated by JA, differentially affecting different members of the plant-associated insect community.

The possiblity exists that JA also regulates the defense of pepper, indirectly, by influencing herbivore-induced plant volatiles, thereby mediating the attraction of carnivorous arthropods, such as parasitoids or predators of thrips, since the importance of JA in attraction of carnivorous enemies of herbivores has been reported for several plant species (Dicke *et al.*, 1999; Gols *et al.*, 2003; Halitschke *et al.*, 2008; Ozawa *et al.*, 2008). The *CaLOX2*-silenced plants will be useful to investigate the role of this gene in indirect defense of sweet pepper.

Conclusion

This study has shown that gene silencing by VIGS is a useful method to functionally characterize candidate genes of pepper for their role in resistance to insects. Through a multidisciplinary approach, involving *in-silico,* transcriptional, chemical, behavioral studies and bioassays, to assess plant resistance, this study identified *CaLOX2* as being involved in the JA-biosynthetic pathway. Moreover, JA-dependent signaling was shown to be important in defense of pepper plants to thrips. Thus, this method allows for investigating functional roles of *C. annuum* genes in plant-insect interactions.

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Chapter 4

Whole-genome transcriptional reprogramming of sweet pepper in response to Western flower thrips feeding

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Abstract

Plants respond to insect herbivory with extensively reprogramming their transcriptome. This dynamic process shapes downstream phenotypic changes. To capture the details of this dynamic transcriptomic response, high-density RNA-Seq analysis of the onset of the response is a powerful tool. We performed a high-density time-series RNA-Seq analysis on leaf tissue of sweet-pepper plants at 12 time points within the first 9 hours of feeding by the cell-content-feeding Western flower thrips (*Frankliniella occidentalis*).Over 3000 pepper genes (~8.6% of the pepper transcriptome) responded to thrips feeding, representing 23 distinct co-expressed gene clusters, 16 up-regulated and 7 down-regulated. Up-regulation occurred fast and was predominantly associated with defence, while down-regulation was slower and associated with developmental processes. The transcription factor families ERF, MYB, NAC, bHLH and WRKY emerged as major regulators of the sweet-pepper response. Chronology analysis showed sequential activation of genes associated with jasmonic acid and ethylene pathways and with the biosynthesis of defence-related phenylpropanoids, flavonoids and terpenoids.Comparative transcriptomic analysis of sweet pepper and *Arabidopsis* responses to *F. occidentalis* feeding revealed overlapping core and plant species-specific responses. Activation of JA pathway genes is part of the core response, while activation of genes involved in isoprenoid biosynthesis is among the sweet pepper-specific responses.

Keywords: Pepper, thrips, RNA-Seq, transcriptomics, high-resolution time series, defence, phytohormones, secondary metabolites

Introduction

Since 400 million years, plants and herbivorous insects interact (Labandeira, 2007). The total number of herbivorous insect species is estimated to be around three million (Schoonhoven *et al.*, 2005). Based on the feeding guild, herbivorous insects can be broadly classified into chewers, phloem feeders and cell-content feeders (Stam *et al.*, 2014). To protect themselves against herbivorous insects, plants have evolved a plethora of direct and indirect defence mechanisms. In direct defence, plant traits influence the performance of herbivorous insects, e.g. by the production of secondary metabolites that influence herbivore growth or mortality (Howe & Jander, 2008; War *et al.*, 2012). In contrast, indirect defence of plants promotes the effectiveness of the enemies of herbivorous insects. Indirect defence may include the release of a cocktail of volatile compounds, so-called herbivore-induced plant volatiles (HIPVs), that attract predators and parasitoids that attack the herbivores (Dicke, 2009; McCormick *et al.*, 2012; Mithofer & Boland, 2012; Dicke, 2015).

Plants recognize herbivorous insects through herbivore-associated molecular patterns (HAMPs) or damage-associated molecular patterns (DAMPs) (Mithofer & Boland, 2008; Bonaventure, 2012; Heidel-Fischer *et al.*, 2014; Duran-Flores & Heil, 2016). Upon herbivore perception, signal-transduction pathways are activated that underlie the induction of defences. This includes an extensive, dynamic transcriptional reorganisation (Kessler & Baldwin, 2002; De Vos *et al.*, 2005; Howe & Jander, 2008; Bidart-Bouzat & Kliebenstein, 2011; Bonnet *et al.*, 2017; Hickman *et al.*, 2017). This complex transcriptional response includes the up-regulation and down-regulation of large numbers of genes, including genes encoding enzymes of phytohormone biosynthetic and response pathways, biosynthetic pathways of primary and secondary metabolites, developmental processes, as well as genes encoding transcription factors. The transcriptional response consists of a plethora of responses with different temporal patterns (Breeze *et al.*, 2011; Windram *et al.*, 2012; Hickman *et al.*, 2017). This complex of temporal transcriptional responses orchestrates the activation and attenuation of various processes, influencing the plant's phenotype. Important progress has been made in analysing the temporally dynamic transcriptomic responses of *Arabidopsis thaliana* to insect herbivory (De Vos *et al.*, 2005; Bidart-Bouzat & Kliebenstein, 2011; Coolen *et al.*, 2016; Davila Olivas *et al.*, 2016) but only limited information is available for other plant species (Bonnet *et al.*, 2017; Durrant *et al.*, 2017). Most of these studies address chewing and phloem-sucking herbivores, whereas information on cell-content feeders is limited.

An early component of phenotypic reprogramming in response to attack involves the accumulation of and changed sensitivity to phytohormones, such as jasmonic acid

(JA), salicylic acid (SA), and ethylene (ET) that regulate plant defence responses (Maffei *et al.*, 2007; Verhage *et al.*, 2010; Erb *et al.*, 2012; Pieterse *et al.*, 2012; Stam *et al.*, 2014). For instance, SA is especially induced by phloem-feeding insects like aphids (Zhu-Salzman *et al.*, 2004; Walling, 2008; Pieterse *et al.*, 2012; Tzin *et al.*, 2015), whereas JA is induced by chewers and cell-content feeders like caterpillars and thrips, respectively (De Vos *et al.*, 2005; Abe *et al.*, 2008; Abe *et al.*, 2009; Pieterse *et al.*, 2012). Moreover, other major phytohormones like abscisic acid, cytokinins, auxin and gibberellins are also reported to act in herbivore-induced defence mechanisms (Pieterse *et al.*, 2012; Stam *et al.*, 2014). JA is a prominent phytohormone modulating induced plant defences against thrips feeding (De Vos *et al.*, 2005; Abe *et al.*, 2008). A microarray-based whole-genome transcriptome study of *Arabidopsis* (3 time points over 72 h) showed that 69% of the thrips-responsive genes were JA responsive (De Vos *et al.*, 2005). In tomato, reduced resistance was observed in the JA-defective mutant *Defenceless1* (*Def1*) and in and *Arabidopsis* in the JA-insensitive *coi1-1* mutant (Li *et al.*, 2002; Escobar-Bravo *et al.*, 2017). In *Arabidopsis* and Chinese cabbage (*Brassica rapa* subsp*. pekinensis*), feeding by the cell-content feeding generalist insect herbivore Western flower thrips (WFT; *Frankliniella occidentalis*) (Pergande) (Thysanoptera: Thripidae) induced the expression of JA-related marker genes, resulting in elevated levels of JA (Abe *et al.*, 2008; Abe *et al.*, 2009). Moreover, exogenous application of JA on several crops resulted in elevated resistance to thrips feeding (Omer *et al.*, 2001; Thaler *et al.*, 2001; Selig *et al.*, 2016).

Evidence from several whole-genome transcriptome studies shows that plants differentially respond to different environmental stresses with a high degree of specificity. Several studies have shown how *Arabidopsis* specifically rearranges its transcriptome over time against different biotic and abiotic stresses (De Vos *et al.*, 2005; Breeze *et al.*, 2011; Windram *et al.*, 2012; Coolen *et al.*, 2016; Davila Olivas *et al.*, 2016; Hickman *et al.*, 2017). In plant-herbivore interactions, the majority of transcriptomic reconfigurations occurs early (minutes to hours) during the interaction, shaping the subsequent plant response to herbivory (Maffei *et al.*, 2007; Stam *et al.*, 2014).

WFT is a devastating pest insect hampering pepper yield worldwide. Thrips are minute cell-content feeding insects exerting direct damage to plants by piercing into epidermal cells and ingesting the contents of mesophyll cells. Their feeding damage results in reflective ‘silver scars’ on plant tissues hampering plant photosynthetic capacity, growth, reproduction and eventually yield (Steiner, 1990; Welter *et al.*, 1990; Shipp *et al.*, 1998; Steenbergen *et al.*, 2018). This herbivore is challenging to control with pesticides due to its thigmokinetic life-style and extensive resistance to pesticides. Additionally, WFT causes indirect damage by acting as vector of tospoviruses, such as *Tomato spotted wilt virus* (TSWV) (Maris *et al.*, 2003). Therefore, to develop thrips-resistant crop varieties to minimize the damage, exploration and understanding

of the genetic basis underlying plant defence responses is vital (Steenbergen *et al.*, 2018). Such crop varieties may be a valuable component of integrated pest management, in combination with biological control (Mouden *et al.*, 2017).

Here, we present an in-depth analysis of the dynamic transcriptomic response of sweet-pepper plants to feeding by WFT through a high-resolution RNA-Seq analysis of the early temporal response. We implemented a state-of-the-art bioinformatics approach to gain in-depth insights into the transcriptomic response of sweet pepper. The key objectives of this study were: (1) To assess the temporal transcriptional reprogramming of sweet pepper in response to WFT feeding, (2) to identify co-expressed gene clusters and their involvement in biological pathways, (3) to identify the major transcription factor (TF) families involved and their binding motifs to unravel directional regulatory connections with downstream regulated genes and their involvement in biological processes, (4) to investigate the chronology of phytohormonal and secondary metabolite pathways underlying induced plant defence, (5) to unravel the conservation of induced responses to WFT feeding between *Arabidopsis* and sweet pepper.

Materials and methods

Plants and thrips

Sweet pepper, *Capsicum annuum* (Mandy variety, Rijk Zwaan (De Lier, The Netherlands)), seeds were sown in 12 cm pots in a greenhouse at 23-25°C, 16L:8D photoperiod and 70±10 % relative humidity. Two weeks later, plants were individually transplanted into 14 cm diameter pots and transferred to a greenhouse with controlled conditions (16L:8D photoperiod, 60 ± 10% relative humidity, 23 ± 5°C diurnal and 20 ± 5°C nocturnal temperatures). Four-week-old sweet pepper plants were used for experiments. Adult females of Western flower thrips *(Frankliniella occidentalis)* reared on chrysanthemum plants were collected and transferred to bean pods (*Phaseolus vulgaris*) in glass jars (10 cm diameter). The jars were incubated in a climate-controlled cabinet with 16L:8D photoperiod, 25±2°C and 70±10 % relative humidity to produce larvae for the experiments (Sarde *et al.*, 2018a; Sarde *et al.*, 2018b).

RNA-Seq experimental setup

Treatment and sampling

Four-week-old sweet pepper plants (having four fully expanded leaves) were each infested with five second instar (L2) WFT larvae, confined in a clip cage (3 cm diameter) on one of the first two leaves. Empty clip cages were used as mock treatment. For each time point and treatment the leaf area in the clip cage (for both, mock and

thrips treatment) was harvested using a cork borer (3 cm diameter), snap frozen in liquid nitrogen and stored at -80 °C; three biological replicates were collected, each biological replicate consisting of one individual plant. Time points of sampling were 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 h of infestation.

RNA extraction and library preparation

RNA extraction was performed using the Qiagen RNeasy Plant Mini Kit including DNase I treatment, following the company's instructions. RNA quantity was assessed using Nanodrop. RNA quality was assessed using RNA Integrity Number (RIN) with Agilent 2100 bioanalyzer. For RNA library preparation, samples with RIN values ≥ 7 were used.

For preparation of the RNA-Seq library and subsequent sequencing, samples were processed according to the TruSeq Stranded mRNA HT Sample Prep Kit from Illumina (Illumina Inc., San Diego, CA, USA). This protocol allows the identification of strand-specific transcripts. Samples were sequenced with an Illumina Hi-seq 2000 platform. Samples were randomly assigned to seven lanes of the Illumina flow cells within each run.

Analysis of RNA-Seq dataset

Processing of raw RNA-Seq data

The RNA-Seq raw reads were subjected to quality control with the FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) tool. Trimmomatic was used to trim the initial 14-basepairs (bp) and Illumina adapters. Reads below 25 bp length were excluded from analysis for all samples (Bolger *et al.*, 2014).

Alignment and quantification of RNA-Seq data

Trimmed RNA-Seq reads were aligned to the *Capsicum annuum* L. Zunla genome using TopHat (v2.0.14) (Kim *et al.*, 2013) with the following parameters: 'p 4', '--min-intron-length 40', '--max-intron-length 2000', '--bowtie-n', '-N 4', '--read-gap-length 2', '--read-edit-dist 4', '--no-novel-juncs'. The aligned reads to each *C. annuum* Zunla gene model were summarized using HTSeq-count (v.0.9.1) (Anders *et al.*, 2015) with parameters: '--stranded no', '-i ID', '–t mRNA'. Principal component analysis (PCA) was performed using the DESeq2 package on regularized $\log_2$-transformed data (Love *et al.*, 2014; Love *et al.*, 2015) in R (https://www.r-project.org/).

Differential gene expression analysis

Differential gene expression (DEG) analysis was executed with the DESeq2 Bioconductor package (Love *et al.*, 2014; Love *et al.*, 2015) in R. Prior to analysis, raw read counts were normalized for sequencing depth across all samples using DESeq2's

median-count normalization procedure. To identify genes that were differentially expressed between mock and thrips-treated plants we used a negative binomial likelihood ratio test (nbinomLRT) considering treatment and time post treatment as factors. Genes with Bonferroni corrected *P* value < 0.01, $\log_2$-fold change ≥ 0.5 or ≤ - 0.5 at one or more time points and read counts ≥ 20 at least in one sample were considered differentially expressed.

Clustering of differentially expressed genes

Temporal expression profiles of the DEGs were clustered using SplineCluster (Heard *et al.*, 2006). For this, $\log_2$-fold change profiles of DEGs at each time point (thrips-treated versus mock), were used with a prior precision stringency of 10^{-4}, cluster reallocation step and the default normalization procedure (Heard, 2011). Default settings were maintained for all other optional parameters.

TF family and promoter motif enrichment analyses

To determine which TF families were enriched among the genes differentially expressed in response to thrips feeding, we tested for overrepresentation of genes encoding members of different TF families found in sweet pepper. Previously, the *C. annuum* Zunla TF families were determined using *C. annuum* cv. CM334 TFs (1665 TFs) from the Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/index.php) using blastp with stringency E-value < 10^{-4}. Overrepresentation of TF families within a set of genes was analyzed using the hypergeometric distribution. *P* values were corrected for multiple testing with the Bonferroni method.

For the promoter motif analysis, characterized *Arabidopsis* TF DNA-binding motifs were retrieved from CIS-DB version 1.02 (Weirauch *et al.*, 2014) and those described in Franco-Zorrilla *et al.* (2014). Promoter sequences defined as the 500 bp upstream of the translation start site were retrieved for all *C. annuum* Zunla genes. The occurrence of a motif within a promoter was determined using FIMO (Grant *et al.*, 2011), where a promoter was considered to contain a motif if it had at least one match with a *P* value < 10^{-4}. Motif enrichment was assessed using the hypergeometric distribution against the background of all sweet pepper genes.

Identification of chronology of defence pathways upon thrips feeding

To identify the chronology of defence pathways in sweet pepper leaves upon thrips feeding, we performed a pairwise comparison between mock and thrips-treated samples at each time point to determine the time point at which DEGs were first differentially expressed (first time of differential expression, *P* < 0.01, $\log_2$-fold change > 0.5 (up-regulated) and < -0.5 (down-regulated) using DESeq2 (Love *et al.*, 2014; Love *et al.*, 2015). For the small number of genes that did not meet these criteria, the time point of first differential expression was defined by minimal *P* value.

Gene Ontology (GO) annotation of the C. annuum Zunla variety

For GO annotation of the *C. annuum* Zunla proteome, we implemented a comparative genomics approach on the *C. annuum* Zunla proteome (Qin *et al.*, 2014) using the GO-annotated *C. annuum* cv. CM334 proteome (Plaza database, https://bioinformatics.psb.ugent.be/plaza/) as query (Kim *et al.*, 2014).

Gene Ontology analysis

For *Arabidopsis* and sweet pepper, GO enrichment analysis was performed using Cytoscape (Shannon *et al.*, 2003) and GOAtools (v0.7.9) (python-based library), using Fisher's exact test (Klopfenstein *et al.*, 2018), respectively. Overrepresentation of the GO categories "biological process", "cellular component" and "molecular function" were determined at $P < 0.05$.

Comparative transcriptomics

The Bi-directional Best Hit (BBH) approach was used on TAIR10 *Arabidopsis* and *C. annuum* Zunla proteome using reciprocal blastp with stringency E-value < 10^{-4} to determine one-to-one orthologues (Martel *et al.*, 2015). The one-to-one orthologue files were processed and used to investigate commonalities and differences between the *Arabidopsis* (Steenbergen *et al.,* in prep.) and *C. annuum* transcriptomes as affected by WTF feeding.

Results

High-resolution transcriptome dynamics in sweet pepper induced by WFT

To comprehensively and accurately capture the dynamics of the full-genome transcriptional response triggered by thrips feeding in sweet pepper plants, RNA-Seq analysis was carried out for thrips-infested and non-infested plants. Initially, samples from 13 time points were sequenced: three replicates of an initial 0h time point plus one replicate for the other 12 time points (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 h of infestation) after thrips infestation. The dataset generated was subjected to sample-to-sample distance analysis and PCA, to capture the transcriptome-level relatedness and variation, respectively, within the samples. The data show an increasing difference between the samples with increasing time after the start of thrips infestation, with exception of the 3h time point (PCA, Fig. S1A). Likewise, the sample-to-sample distance heat map (Fig. S1B) clearly shows the close relatedness between sequential time points. Both analyses complement each other and show that the transcriptional response gradually developed over time. Based on this analysis, the 1, 2, 3, 4, 6 and 8 h time points were selected because they captured the full dynamic range of the transcriptional response to thrips feeding.

To understand the effect of the factors treatment and time on the transcriptomic profile of sweet pepper plants, all three replicates of the selected six time points were subjected to PCA (Fig. 1A). Both treatment and time explicitly separated the transcriptomes. The difference between mock-treated and thrips-infested plants is small at 1h since the initiation of thrips infestation and the difference gradually increases with time. At all six time points the transcriptome of thrips-infested plants is clearly different from that of mock-treated plants. The mock-treated samples exhibit a change in transcriptome with time, suggestive of an effect of circadian rhythm on the transcriptome of sweet pepper plants. Variation within the replicates of thrips-treated samples is higher than for mock-treated samples, which may represent differences in the intensity of thrips feeding among replicates.

Genes that are differentially expressed in thrips-infested and uninfested plants at different time points were identified with a generalized linear model (GLM) using the DESeq2 (Love *et al.*, 2014). This analysis identified a total of 3062 differentially expressed genes (DEGs) (Supplemental Data Set 1), that represent 8.6% of the total set of pepper transcripts (35,336) (Qin *et al.*, 2014). The JA-biosynthesis genes *CaLOX2* and *CaAOS*, and the JA-regulated genes *CaMYC2*, *CaPIN II* are among the DEGs. These representative genes of the JA response show a clear up-regulation within 1-2 h (Fig. 1B), thus supporting the JA-component of the response of sweet pepper to infestation by WFT (Sarde *et al.*, 2018a).

Cluster analysis of DEGs based on expression patterns

To identify and classify clusters of co-expressed genes responding to thrips feeding over time, the time-series clustering algorithm SplineCluster was employed. A total of 23 clusters, 16 clusters of up-regulated genes and 7 clusters of down-regulated genes (Supplemental Data Set 2) was identified. 2060 DEGs (67.3%) are represented in the sixteen up-regulated clusters (Clusters 1-16) and 1002 DEGs (32.7%) in the seven down-regulated clusters (Clusters 17-23) (Fig. 2). This analysis shows that the pepper transcriptional response to thrips feeding is highly dynamic over time. Many changes are initiated between 1h and 2h after the onset of thrips feeding. The clusters of up-regulated genes show more variation in temporal patterns than the clusters of down-regulated genes (Fig. 2).

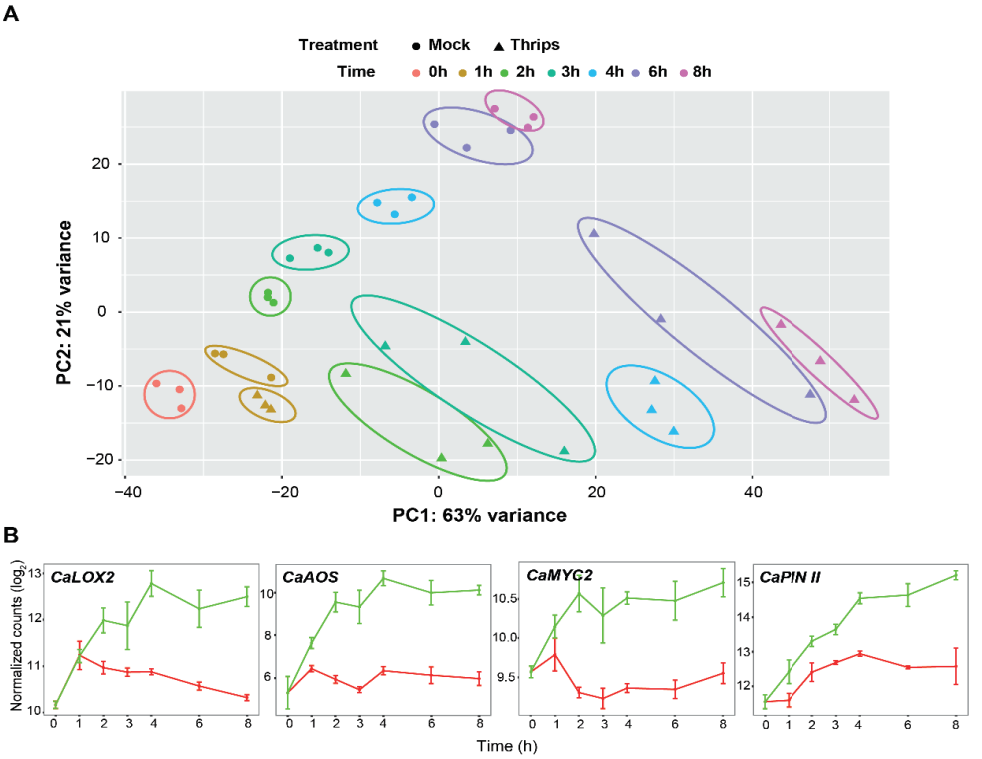


Figure 1. Principal component analysis (PCA) of whole-genome transcriptomic response at different time points for thrips-infested and mock-infested plants plus expression profile of four selected JA-related marker genes of sweet pepper. (A) PCA of sweet pepper transcriptome of non-infested (mock) and infested (thrips) plants; samples were harvested at six time points. PCA was generated on the regularized log2-transformed data within the DESeq2 R package. Colours and symbol shapes indicate time points and treatments, respectively. Variation explained by the two principal components is depicted on both axes. (B) Expression pattern of selected JA-related marker genes from RNA-Seq dataset. Data represent mean ± SE (n = 3 biological replicates). Red and green colours indicate mock and thrips treatment, respectively.

To investigate biological significance of the co-expressed genes per cluster, we explored overrepresentation of Gene Ontology (GO) terms for the genes per cluster with the python-based library GOAtools (v0.7.9) (Klopfenstein *et al.*, 2018). Each cluster is overrepresented for several functional terms, including unique and common categories (Fig. 2B) (Supplemental Data Sets 3 and 4). As predicted, defence-related and JA-related functional categories are overrepresented in several up-regulated gene clusters. For instance, “response to wounding” is overrepresented in clusters 6 and 11; “response to JA” is overrepresented in clusters 4 and 7, and “defence response to insects” in cluster 11. In cluster 5, “response to salicylic acid” is overrepresented, suggesting that besides JA-related processes also SA-related processes play

a role in the response of pepper to thrips feeding. Moreover, several up-regulated clusters also exhibited overrepresentation of more unique functional categories. For instance, cluster 1 was specifically enriched for the GO term “tryptophan metabolic process”, which is associated with the production of defensive secondary metabolites (Kang & Back, 2006; Hiruma *et al*., 2013)and cluster 2 for “terpene/sesquiterpene biosynthetic process”, compounds involved in direct and indirect defence against insects (Gershenzon & Dudareva, 2007) (Supplemental Data Set 3). Down-regulated clusters were associated with GO annotations like “response to auxin”, “response to high light”, suggesting that plant processes related to growth and development are down-regulated in response to thrips feeding (Supplemental Data Set 4). Taken together, this analysis shows that up-regulated clusters are enriched with genes annotated for being involved in defence-related responses, whereas down-regulated clusters are enriched with genes annotated for being involved in developmental processes. Some clusters harbour genes that are specifically enriched in a biological process that is not represented by other clusters, while especially JA-associated processes are commonly enriched by several clusters.

TF family abundance and TF binding motif analysis

TFs are important regulators of transcriptional responses and, thus, of the resulting phenotypic change. They bind to DNA-regulatory sequences in the promoter regions of target genes contributing to modulation of gene expression. The investigation of *C. annuum* Zunla TF families using 1665 TFs of *C. annuum* cv. CM334 identified a total 1424 (unique or non-redundant) TFs in *C. annuum* Zunla (Supplemental Data Set 5). To gain insight in regulators of the pepper transcriptome in response to thrips infestation, we analysed TF family abundance in all up- (2060) and down-regulated (1002) genes. Several TFs belonging to families like ERF, MYB, NAC, bHLH and WRKY are significantly overrepresented among the up-regulated genes (Fig. 3A), suggesting a pivotal role of these TF families in regulating plant responses in sweet pepper plants that are induced upon thrips feeding. Among the down-regulated genes, the TF families HD-ZIP and bHLH are overrepresented.

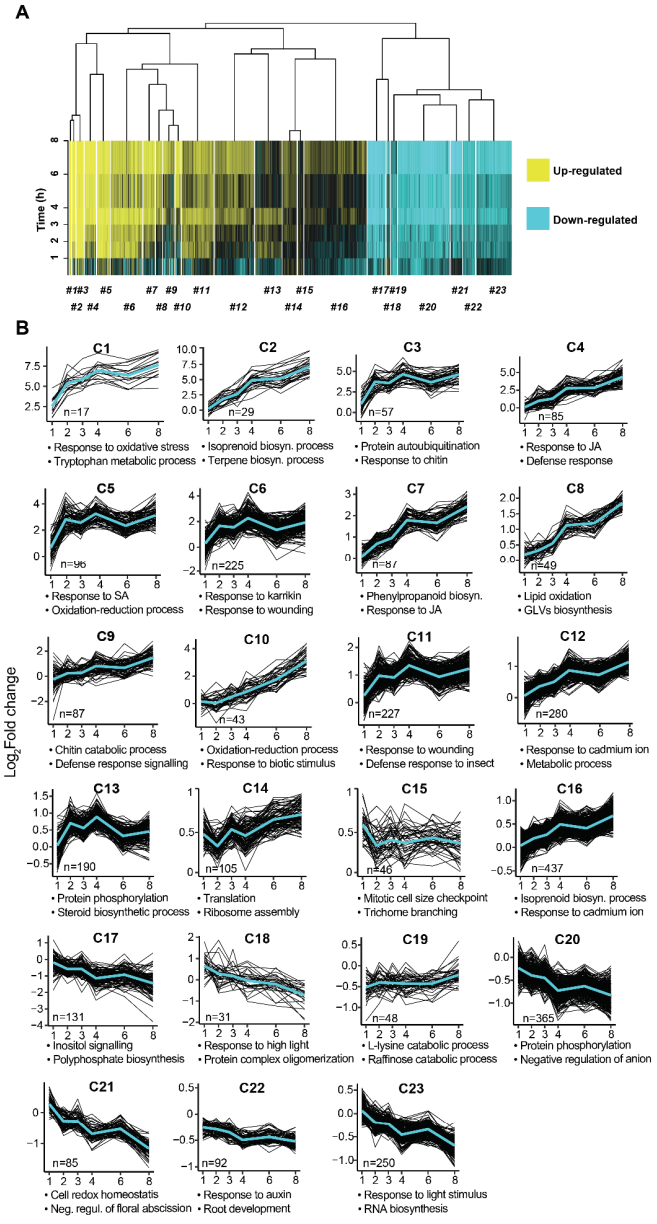


Figure 2. **Co-expressed gene clusters of differentially expressed genes in response to feeding by *Frankliniella occidentalis*.** SplineCluster analysis was performed using log_2-transformed expression ratios (thrips / mock). **(A)** Heat map depicting expression profile of 3062 DEGs over time in each cluster. Cluster numbers are depicted with '#' below the heat map. Yellow and blue indicate up- and down-regulated genes, respectively. **(B)** Twenty-three partitioned gene clusters (1-16 up- and 17-23 down-regulated) with selected enriched GO-terms. The mean expression profile of each cluster is represented by the blue line. The x-axis represents expression pattern of each gene cluster over time (in hours) and y-axis represents log_2fold change of genes from each cluster.

Because TF binding specificities are typically conserved between related organisms (Portales-Casamar *et al.*, 2010), we extended our analysis of transcriptional regulation underlying the pepper response to thrips by utilizing recently identified DNA binding motifs for 580 *Arabidopsis* TFs (Franco-Zorrilla *et al.*, 2014; Weirauch *et al.*, 2014), and scanned the promoter sequences of the genes in all 23 co-expressed clusters for overrepresentation of TF binding motifs. In clusters representing up-regulated genes, promoter regions were especially enriched with binding sites for bHLH, ERF, bZIP and WRKY TFs (Fig. 3B). For example, bHLH motifs were particularly enriched in clusters 4 and 7, ERF motifs in clusters 14 and 16, bZIP motifs in clusters 5 and 11 and WRKY motifs in clusters 6 and 16. Motifs that correspond to bHLH and WRKY TF binding sites were overrepresented in clusters involved in JA-related processes (clusters 4 and 7; Supplemental Data Set 3) and SA-related responses (cluster 6; Supplemental Data Set 3), respectively. In down-regulated clusters, Dof, MIKC, MYB-related and TCP TF-specific binding sites are overrepresented (Fig. 3B). In contrast to TF abundance analysis (Fig. 3A), no enrichment of bHLH binding motifs was found in genes of down-regulated clusters (Fig. 3B). Collectively, the expression profiles of pepper TFs and the enrichment of their binding sites in the thrips-induced pepper transcriptome provides important insight into the architecture of the gene regulatory network of pepper in response to thrips infestation.

Chronology of sweet pepper defence response upon thrips feeding

The transcriptional response to thrips feeding develops with time (Fig. 1). To understand the chronology of biological processes activated upon thrips feeding, we performed a pairwise comparison (mock versus thrips-treated) for each time point on the temporal RNA-Seq dataset (Fig. 4). This analysis highlights the dynamic transcriptome profile and distinguishes DEGs (up- and down-regulated) that are differentially expressed for the first time (**f**irst **t**ime **o**f **d**ifferential **e**xpression (ftode)) and DEGs that become **a**gain **d**ifferentially **e**xpressed (ade) (Fig. 4) (Supplemental Data Set 6). At 2h after the introduction of thrips ca 10 times more genes are up-regulated than down-regulated. The majority of DEGs (up- and down-regulated) became first differentially expressed within 4h and 8h since the onset of thrips feeding with twice as many ftode genes being up-regulated compared to ftode genes being down-regulated.

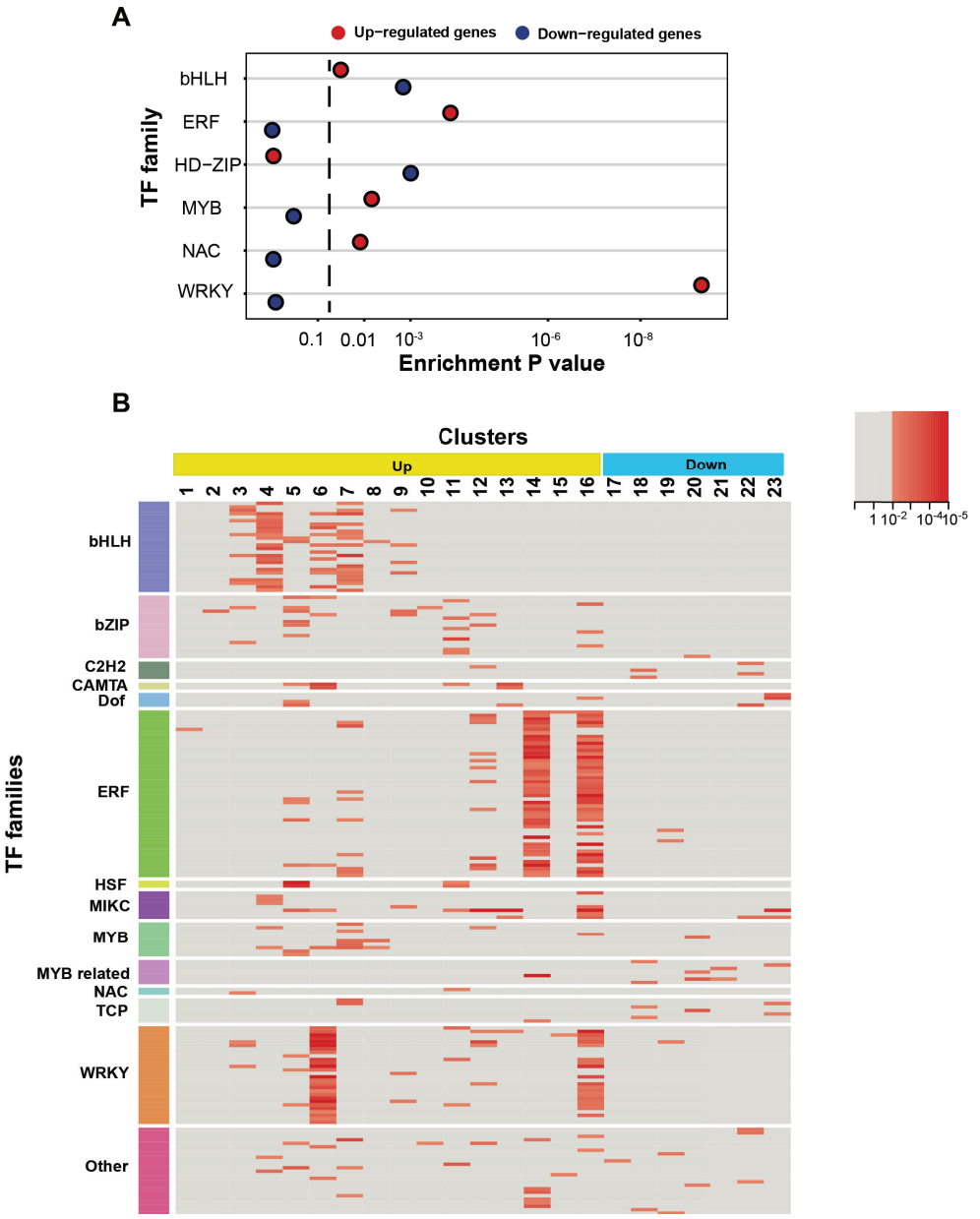
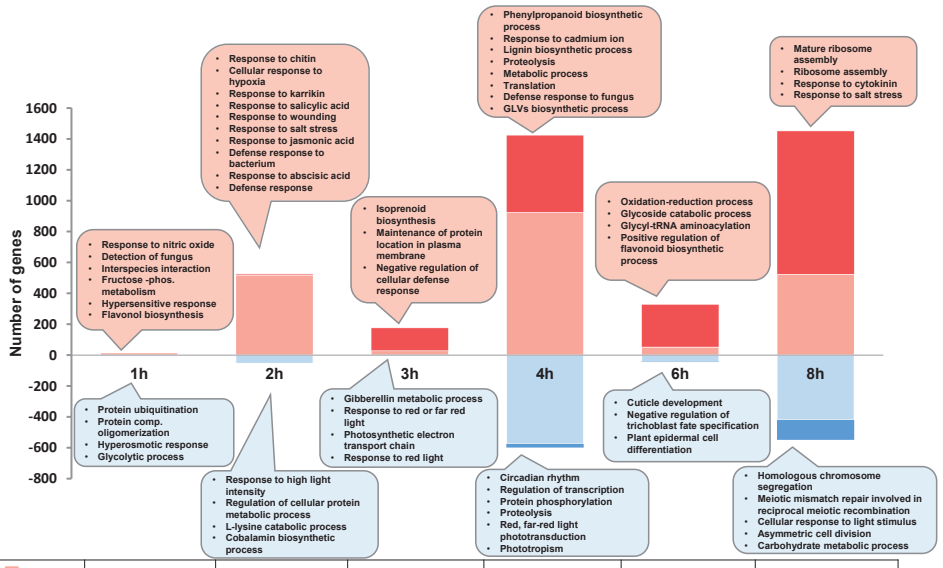


Figure 3. Overrepresented TF families and enriched TF DNA binding motifs in differentially expressed gene clusters. (A) TF families among up-regulated (red) and down-regulated (blue) genes upon WFT feeding. Black dotted line represents significance threshold ($P < 0.05$). **(B)** Enriched TF DNA binding motifs in promotors of genes in up- and down-regulated gene clusters. The red colour intensity corresponds to raw *P* value of enriched motifs. Columns and rows represent cluster numbers and enriched motifs, respectively. Clusters 1-16 are up-regulated and clusters 17-23 are down-regulated.



	1h	2h	3h	4h	6h	8h
Up (ftode)	14	519	28	924	52	523
Up (ade)	0	7	148	500	277	929
Down (ftode)	7	54	4	475	42	420
Down (ade)	0	0	0	24	3	129

Figure 4. Chronology of sweet pepper transcriptome profile and defence pathways on Western flower thrips feeding. Number of genes responding at each time point are depicted in the legend under the figure. Selected GO terms for the first time of differential expression (ftode) genes are shown for each time point. Light and dark blue colours depict up-regulated first time of differentially expressed (ftode) genes and again differentially expressed (ade) genes, respectively. Light and dark orange depicts down-regulated first time of differentially expressed (ftode) genes and again differentially expressed (ade) genes.

Among the up-regulated genes at the 2h time point, GO terms are mainly associated with hormonal and defence responses, reflecting the induction phase of the defence response to thrips infestation. Likewise, at the 4h time point, genes associated with GO terms like “phenylpropanoid biosynthetic process”, “lignin biosynthetic process”, “green leaf volatile biosynthetic process” are induced, reflecting the onset of direct (phenylpropanoids, lignin) and indirect (green leaf volatiles, terpenoids) defence activation. Among the down-regulated genes, GO terms are associated with growth and development (“gibberellin metabolic processes”, “response to red or far red light”, “photosynthetic electron transport chain”). Overall, this analysis indicates that the up-regulation of genes in response to thrips infestation occurs faster and more intensively than the down-regulation of genes. Apparently activation of defence-related processes is prioritized over attenuation of growth- and development-related processes.

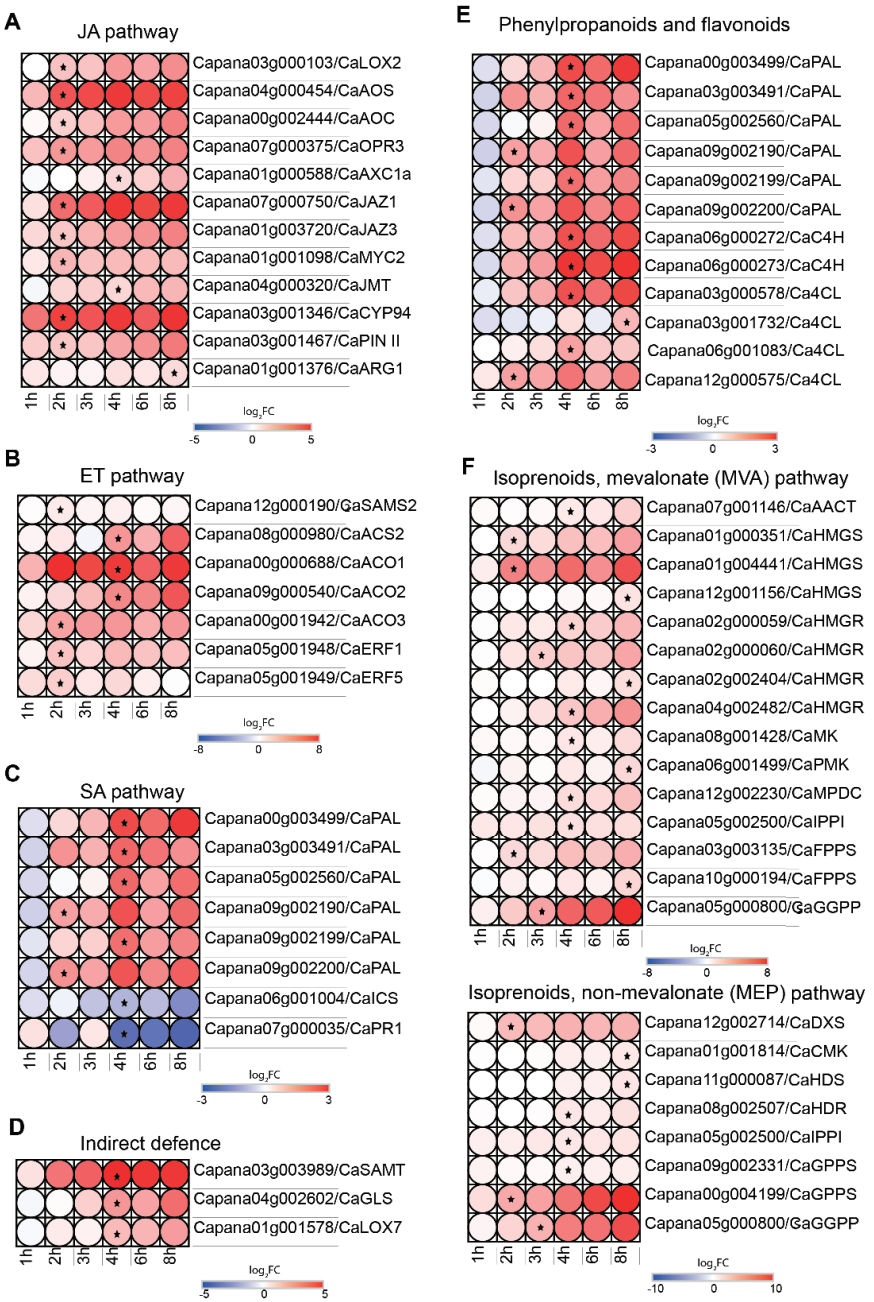


Figure 5. Expression of genes involved in defence-related hormonal cascades, indirect defences and biosynthesis of secondary metabolites. (A) JA pathway, **(B)** ET pathway, **(C)** SA pathway, **(D)** Indirect defence, **(E)** phenylpropanoid and flavonoid pathways and **(F)** Isoprenoid pathways (MVA and MEP pathways). ★ indicates significant ($P < 0.01$) first time of differential expression (ftode) of each gene.

Transcription dynamics of genes involved in hormonal and secondary metabolite pathways

To dissect the temporal response of several hormonal and metabolic pathways to thrips feeding, we focused on the expression of genes involved in these pathways. The majority of genes in the JA biosynthetic pathway (*CaLOX2*, *CaAOS*, *CaAOC*, *CaOPR3*, *CaJAZ1*, *CaJAZ3*, *CaMYC2*, *CaCYP94*, *CaPIN II*, *CaARG1*) are significantly induced at 2h after the introduction of thrips onto the plant and sustain up-regulated until the last time point assessed, i.e. 8h after introduction of thrips (Fig. 5A). Likewise, several genes associated with the ET pathway (*CaSAMS2*, *CaACO3, CaERF1*, *CaERF5*) are up-regulated at the 2h time point (Fig. 5B). SA biosynthesis in plants can occur via two pathways: the isochorismate synthase (ICS) and phenyl alanine lyase (PAL) pathways; depending on the plant species the induced accumulation of SA can occur primarily through either pathway (Chen *et al.*, 2009). Several homologues of *CaPAL* are up-regulated at the 4h time point, while *CaICS* and the SA-responsive gene *CaPR1* are down-regulated at this time point (Fig. 5C). Furthermore, the genes and their homologues involved in phenylpropanoid and flavonoid biosynthesis (*Ca4CL*, *CaC4H*), including *CaPAL* homologs, are up-regulated at 4h after thrips introduction (Fig. 5E). For the biosynthesis of terpenoids (isoprenoids), an important class of VOCs involved in indirect plant defense in many plant species including pepper (Dicke *et al.*, 1990; Van Den Boom *et al.*, 2004), several genes involved in the MVA (mevalonate) and MEP (methylerythritol 4-phosphate) pathways are significantly induced within 3-4h after introduction of thrips (Fig. 5F). Similarly, other genes involved in the production of VOCs, for example, geranyllinalool synthase (*CaGLS*) involved in biosynthesis of terpenes, methyl salicylate (MeSA) (*CaSAMT*) and green leaf volatiles (GLVs) (*CaLOX7*) were induced after 4h of thrips feeding (Fig. 5D). Taken together, the JA and ET pathways were rapidly up-regulated (within 2h), the SA pathway seems down-regulated, whereas genes involved in secondary metabolism and indirect defence are induced at 4h after the onset of thrips feeding.

Conservation of defence responses between *Arabidopsis* and sweet pepper upon WFT feeding

A similar RNA-Seq analysis of the dynamic response to feeding by *F. occidentalis* has been performed for *Arabidopsis,* identifying in total 2788 DEGs (1820 up- and 968 down-regulated) for eleven time points distributed over 8 h of thrips feeding (Steenbergen et al., *in prep*). In the present study we have identified 3062 DEGs (2060 up- and 1002 down-regulated) for six time points distributed over 8 h of thrips feeding on pepper. To gain insight in commonalities and differences in the transcriptomic responses of sweet pepper and *Arabidopsis*, we compared the DEGs recorded in these two studies. In pepper, 981of the 2060 up-regulated genes and 469 of the 1002

down-regulated genes, respectively, have *Arabidopsis* orthologues (Supplementary Data Set 7). In *Arabidopsis*, 652 of the 1820 up-regulated genes and 392 of the 968 down-regulated genes, respectively, have pepper orthologues. Thus, there is ca 35-50% overlap in transcriptomic responses of sweet pepper and *Arabidopsis* to feeding by *F. occidentalis.* The majority of DEGs from both plants having orthologues in the other plant species are differentially expressed in a species-specific manner: 718 up-regulated plus 405 down-regulated genes in sweet pepper and 393 up-regulated plus 324 down-regulated in *Arabidopsis* (Fig. 6). There was more overlap in orthologous genes up-regulated in both plant species (232, 24% of up-regulated pepper DEGs with *Arabidopsis* homologues) than in orthologous genes down-regulated in both species (37, 8% of down-regulated pepper DEGs with *Arabidopsis* homologues) (Fig. 6). Moreover, there is limited overlap in contrasting responses of orthologous genes, i.e. genes that are up-regulated in pepper and down-regulated in *Arabidopsis* (31) and vice-versa genes that are down-regulated in pepper and up-regulated in *Arabidopsis* (27). Genes from each subset are listed in Supplemental Data Set 7.

GO-term analysis was carried out to assess which processes have similar, specific or dissimilar regulation in the two plant species (Fig. 6). Common up-regulated DEGs (232) are associated with GO terms like “JA biosynthetic process” and several other defence-related processes, highlighting the important role of the JA pathway in both plants against WFT. DEGs in sweet pepper that have orthologues in *Arabidopsis* and that are up-regulated in pepper and not in *Arabidopsis* (718) are associated with GO terms like “isoprenoid biosynthetic process”, “response to cadmium ion”, whereas up-regulated genes in *Arabidopsis* that have orthologues in sweet pepper (393) are associated with GO terms like “response to stress”, “response to biotic stimulus”, “response to endogenous stimulus”, reflecting defence responses that are specifically up-regulated in *Arabidopsis* and not in sweet pepper. In pepper, up-regulated DEGs not having orthologues in *Arabidopsis* (1079) exhibit an overrepresentation of the GO term “response to jasmonic acid”, suggesting the existence of a unique downstream JA response in pepper. Moreover, among the common down-regulated genes (37), a high overrepresentation of genes involved in “spindle organization”, “monopolar cell growth” and “transpiration” was recorded. Similarly, several common and unique GO terms for each subset of down-regulated genes are highlighted (Fig. 6).

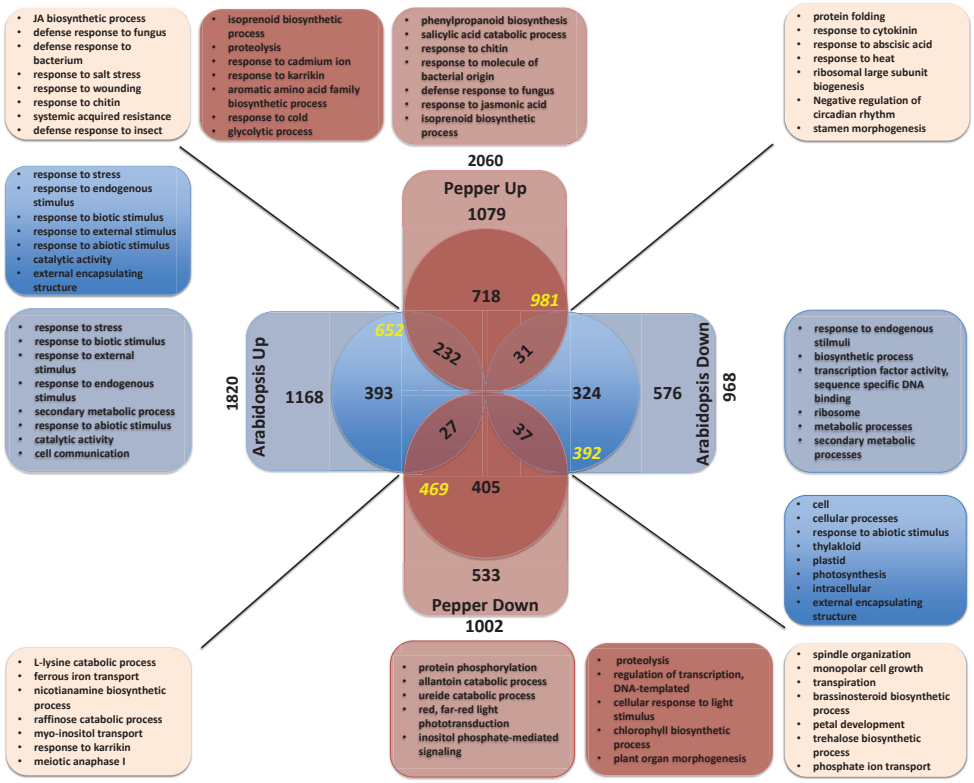


Figure 6. **Venn diagram depicting conservation and uniqueness of induced defences in *Arabidopsis* and sweet pepper upon WFT feeding**. Selected overrepresented GO terms are depicted for each subset of genes. The yellow numbers depicts orthologue number for each subset of genes.

Discussion

Through high-resolution temporal RNA-Seq and subsequent in-depth bioinformatic analysis, our study presents comprehensive temporal insights into sweet pepper transcriptomic responses to WFT feeding at an unprecedented level. This study shows that approximately 10% of the sweet pepper transcripts are involved in temporal transcriptional reprogramming upon thrips feeding. Most of this transcriptional reprogramming is initiated within 4h after the onset of thrips feeding, with up-regulation exhibiting a faster time course than down-regulation. Down-regulated genes are especially involved in developmental processes and up-regulated genes are especially involved in defence mechanisms. The induced transcriptome depicts a dynamic expression pattern over time, consisting of 16 up-regulated gene clusters and comprising genes involved in several hormonal and secondary metabolite pathways. The in-depth bioin-

formatic analysis also provides insight into the underlying regulators (TFs) and chronology of the transcriptomic rearrangement and biological processes affected. Moreover, a comparison with WFT-mediated rearrangement of the *Arabidopsis* transcriptome, indicates a core thrips response, which is early and involves processes related to JA biosynthesis and signalling, and a relatively large plant-species specific response, which includes isoprenoid biosynthetic process. There overlap in the transcriptomic responses of pepper and *Arabidopsis* to WFT is especially exhibited in JA-related processes.

Transcriptional analysis of plant responses to insect herbivory

Transcriptional responses to insect feeding have been carried out with microarrays and RNA-Seq for various plant species, but especially *Arabidopsis.* Full-genome microarray analyses have been carried out for *Arabidopsis* in response to feeding by caterpillars (De Vos *et al.*, 2005; Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011; Appel *et al.*, 2014; Davila Olivas *et al.*, 2016; Kroes *et al.*, 2017), aphids (De Vos *et al.*, 2005; Bidart-Bouzat & Kliebenstein, 2011; Kroes *et al.*, 2017) or whiteflies (Kempema *et al.*, 2007; Zhang *et al.*, 2013). Full-genome microarray or RNA-Seq studies of responses of other plant species to herbivory have been carried out for e.g. caterpillars on wild cabbage (Broekgaarden *et al.*, 2011), aphids on maize (Tzin *et al.*, 2015) spider mites on tomato, maize, barley and grapevine (Martel *et al.*, 2015; Diaz-Riquelme *et al.*, 2016; Bui *et al.*, 2018) or whiteflies on cabbage (Broekgaarden *et al.*, 2018). Caterpillars have a biting-chewing feeding mode, removing sections of a leaf. Aphids and whiteflies have a piercing-sucking feeding mode, and ingest phloem contents. Spider mites have delicate piercing stylets, which they insert in the plant to pierce mesophyll cells to ingest their contents. Thrips also feed on mesophyll cells. They damage a group of cells with their mouthparts and then suck up the contents of the opened cells with their stylets (Steenbergen *et al.*, 2018). To the best of our knowledge there are two published studies that have made a full-genome transcriptomic analysis of plant responses to thrips: alfalfa response to feeding by *Odontothrips loti* (Tu *et al.*, 2018) (RNA-Seq analysis, only one time point) and *Arabidopsis* response to *Frankliniella occidentalis* (De Vos *et al.*, 2005) (microarray analysis, two time points spread over 24h). Although *Arabidopsis* also shows a strong representation of genes involved in JA-related processes among the up-regulated genes (De Vos *et al.*, 2005). The SA pathway seems especially up-regulated in alfalfa (Tu *et al.*, 2018).

Most transcriptomic studies of plant responses to insect feeding have assessed the response at one or two time points that often span a time period of 24 h or longer (De Vos *et al.*, 2005; Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011; Zhang *et al.*, 2013; Appel *et al.*, 2014; Diaz-Riquelme *et al.*, 2016; Kroes *et al.*, 2017; Broekgaarden *et al.*, 2018; Tu *et al.*, 2018). An exception are studies by Coolen *et al.* (2016) who included 4 time points during 24 h of feeding by caterpillars on *Arabidopsis* and

Durrant *et al.* (2017) who assessed the transcriptomic response of wild tobacco to the application of oral secretion of the caterpillar *Manduca sexta* at 6 time points ranging from 0.5 to 13 hours since treatment In the latter study no further feeding damage was done throughout the 13 hours of the experiment. The present study is the first to provide a detailed high-resolution analysis of the early transcriptional response of a plant to a cell-content feeding insect herbivore, and thus provides a valuable resource for investigating this complex interaction. Our data show that with 6 time points in the first 8 h of thrips infestation a total 23 clusters of transcriptional patterns can be distinguished, indicative of the complexity of the transcriptional response to continuing infliction of insect herbivory.

Temporal transcriptomic dynamics of sweet pepper to WFT feeding

Most of the temporal transcriptomic plant responses to insect herbivory are studied with limited time points and not possessing control or mock samples for each time point (De Vos *et al.*, 2005; Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011; Zhang *et al.*, 2013; Appel *et al.*, 2014; Diaz-Riquelme *et al.*, 2016; Kroes *et al.*, 2017; Broekgaarden *et al.*, 2018; Tu *et al.*, 2018). To obtain accurate insights into temporal transcriptome dynamics, it is not only crucial to harvest more time points, but it is also vital to harvest the control or mock samples for each time point (Breeze *et al.*, 2011; Windram *et al.*, 2012; Lewis *et al.*, 2015; Hickman *et al.*, 2017). Harvesting at a limited number of time points will provide a low-resolution temporal transcriptome portrait of the plant response. Moreover, when control or mock samples are not included for each time point an effect of circadian rhythm (Fig. 1A) is not compensated for in the temporal differential gene expression analysis, generating false positives. Therefore, in our study, together with harvesting more time points, inclusion of control samples for each time point was an important feature. Furthermore, overall, sweet pepper responded with a temporally dynamic transcriptome to WFT feeding, including more up-regulated (2060) genes than down-regulated (1002) genes. This temporally dynamic transcriptome shows that numbers of genes induced or repressed vary among different time points, indicating major switch points for up- and down-regulated defence mechanisms at different time scales. This suggests that harvesting tissue at fewer time points bears the risk of missing important temporal dynamics in the transcriptome response.

Role of phytohormones and secondary metabolites in sweet pepper against WFT

Besides the role of JA, relatively little knowledge on other defence components against thrips resistance is available (Maharijaya *et al.*, 2012). JA is a major phytohormone in regulating induced plant defences against thrips (De Vos *et al.*, 2005; Abe *et al.*, 2008). For example, 69% of differentially expressed genes in *Arabidopsis* were JA-re-

lated (De Vos *et al*., 2005). JA-signaling-impaired tomato and pepper plants show enhanced susceptibility to thrips (Li *et al*., 2002; Escobar-Bravo *et al*., 2017; Sarde *et al*., 2018a). Induction of the whole JA cascade in our RNA-Seq dataset validates the conservation of JA-regulated defences in sweet pepper against thrips (Sarde *et al*., 2018a). Some of the JA-regulated proteins like proteinase inhibitors (e.g. encoded by *CaPIN II*) and Arginase (*CaARG1*) are known for antidigestive effects in WFT (Outchkourov *et al*., 2004) or other arthropods (Chen *et al*., 2004). Likewise, up-regulation of several genes associated with the ET pathway may be indicative of synergism between the JA and ET pathways in defence of sweet pepper against thrips as has been shown for other biotic stresses in *Arabiopsis* (Pieterse *et al*., 2009). SA biosynthesis in plants occurs either via the PAL or ICS pathway (Chen *et al*., 2009). Up-regulation of several homologues of *CaPAL* and down-regulation of *CaICS* and the SA-responsive *CaPR1* (Fig. 5C) gene suggest a suppression of SA pathway (at least until 8h), upon thrips feeding. *PAL* genes are also known to be involved in the biosynthesis of phenylpropanoids and flavonoids and their induction does not necessarily mean an induction of the SA pathway. Sarde *et al.* (2018a) also showed that *CaPR1* is induced later i.e. post 10h of thrips feeding, suggesting later activation the SA pathway. Furthermore, up-regulation of phenylpropanoid and flavonoid biosynthetic genes and their homologs (*Ca4CL1*, *CaC4H*), together with *CaPAL* homologues, suggests a defensive role of these pathways against thrips. Moreover, plants also activate indirect defence by emitting VOCs upon herbivory feeding to attract their natural enemies (Mithofer & Boland, 2012; Dicke, 2015). The blend of VOCs emitted mainly comprises of GLVs, methyl salicylate (MeSA) and terpenoids (Dudareva *et al*., 2006; Mumm *et al*., 2008). Terpenoid biosynthesis occurs via the cytosolic MVA (mevalonate) or plastidal MEP (methylerythritol 4-phosphate) pathways (Vranova *et al*., 2013). In this study, together with many MVA and MEP pathway genes, the genes involved in biosynthesis of MeSA (*CaSAMT*), GLVs (*CaLOX7*) (Sarde *et al*., 2018b) and terpenoids (Fig. 5D) are induced. This suggests the emission of a VOC blend similar to that induced by another cell-content feeder, the spider mite *T. urticae* in sweet pepper (Van Den Boom *et al*., 2004).

Cluster analysis identifies major regulators in sweet pepper

Several TF families regulate the transcriptional reprogramming of plants in a stress-specific manner (Breeze *et al*., 2011; Windram *et al*., 2012; Hickman *et al*., 2017; Jin *et al*., 2017). Upon thrips feeding, the TF families ERF, MYB, NAC, bHLH and WRKY appeared to be major regulators in modulating the majority of thrips up-regulated genes. Recently, Hickman *et al.* (2017) showed that the ERF, MYB and bHLH TF families are major regulators of transcriptome reprogramming in response to exogenous MeJA application. This suggests that regulation of JA-signalling in *Arabidopsi*s and sweet pepper is conserved. In contrast, WRKY TFs, known to regu-

late SA-mediated responses (Pandey & Somssich, 2009; Rushton *et al.*, 2010) are overrepresented among the up-regulated genes (Fig. 3A), suggesting an induction of SA responses upon thrips feeding. In contrast, the down-regulation of SA biosynthetic (isochorismate synthase (*CaICS*)) and SA-responsive (*CaPR1*) gene suggests suppression of the SA pathway (at least until 8h) upon thrips feeding. Possibly, the up-regulated WRKY TFs regulate phenylpropanoid, flavonoid or terpene biosynthesis (Schluttenhofer & Yuan, 2015) or SA responses independent of the isochorismate pathway are activated (Chen *et al.*, 2009). Furthermore, in the TF motif analysis, binding sites of bHLH TFs, known to be involved in JA signalling (Goossens *et al.*, 2017), appeared to be enriched in several clusters (Fig. 3B), suggesting that bHLH TFs extensively regulate thrips-induced JA-regulated genes. In contrast, ERFs appeared to be enriched in a few clusters suggesting that they regulate only a small component of the transcriptional response to thrips. Likewise, enrichment of WRKY TFs in clusters overrepresented with SA-responses consolidates its role as pivotal regulator of SA-response pathways (Pandey & Somssich, 2009).

Sequential activation of phytohormones and secondary metabolites

In plant-herbivore interactions, phytohormones are induced early (timescale of minutes to hours) resulting in an activation and regulation of the downstream transcriptome (Maffei *et al.*, 2007). Many studies have shown a reprogramming of the transcriptome occurring in plants with time in response to different stresses (Breeze *et al.*, 2011; Windram *et al.*, 2012; Bechtold *et al.*, 2016; Hickman *et al.*, 2017). This temporal transcriptome reprogramming has consequences for the activation of different metabolic processes (timescale of hours to days) involved in plant defence (Maffei *et al.*, 2007). In the chronology analysis, at the 2h time point, GO terms like “response to JA”, and “response to wounding” are overrepresented. Similarly, genes involved in JA biosynthesis (*CaLOX2*, *CaAOS*, *CaAOC*, *CaOPR3*, *CaJAZ1*, *CaJAZ3*, *CaMYC2*) and ET biosynthesis (*CaSAMS2*, *CaACO3*) (Fig. 4) are differentially expressed for the first time (ftode) at this time point, indicative of activation of the JA and ET hormonal pathways within 2h of thrips feeding. At the 4h time point, overrepresentation of GO terms like “phenylpropanoid biosynthetic process”, “GLVs biosynthetic process” and activation (ftode) of major genes involved in biosynthesis of MeSA (*CaSAMT*) and terpenoids via MVA and MEP (*CaAACT*, *CaHMGR*, *CaMK*, *CaHDR, CaMPDC*, *CaIPPI, CaGLS*) pathways indicates that the induced biosynthesis of these secondary metabolites is initiated at this time point. The major regulator of the most abundant volatile induced in sweet pepper by spider mites, the homoterpene (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), *CaGGPPS*, is activated earlier at 3h time point (Fig. 5F). This fits to the fact that VOCs such as ester methyl salicylate (MeSA), monoterpenes myrcene and β-ocimene, the homoterpene TMTT, and the sesquiterpene (*E,E*)-α- farnesene are emitted hours after herbivorous insect infestation (Erb *et al.*,

2015; Aljbory & Chen, 2018).

Majority of induced defences are plant-species specific against WFT

To gain more insight in conservation and diversification of induced defence mechanisms against WFT between *Arabidopsis* and sweet pepper, we implemented a comparative transcriptomic analysis. From this analysis it appears that more genes, up- as well as down-regulated DEGs are specific for each of the two plant species than common for both of them. There was more overlap in up-regulated DEGs (232) than in down-regulated DEGs (37), which indicates that up-regulated responses such as induced defences are more conserved than down-regulated responses, which concern plant growth and development. The prominent association of a common subset of up-regulated DEGs (232) with the GO term "JA biosynthetic process", comprising mainly JA biosynthetic and signalling genes (*CaLOX2*, *CaAOS*, *CaAOC*, *CaOPR3*, *CaACX1*, *CaJAZ1*, *CaJAZ3*, *CaMYC2*, *CaJMT*) together with other genes involved in ET biosynthesis (*CaSAMS2*), ET response (*CaERF1*, *CaERF5*), phenylpropanoid biosynthesis (*CaC4H*) and indirect defence mechanism (*CaSAMT*, *CaGLS*, *CaLOX7*), indicates a prominent conservation of the JA pathway with other defence mechanisms in *Arabidopsis* and sweet pepper. Furthermore, the common up-regulated DEGs (232) of pepper are associated with the GO term "JA biosynthetic process", whereas the non-orthologous DEGs (1079) from pepper are associated with the GO term "response to JA". This suggests that the JA biosynthetic pathway is conserved, whereas the response to this phytohormone is diverged between the two plant species. One of the JA responsive pathways in Arabidopsis is the glucosinolate pathway, resulting in secondary metabolites that are specific for brassicaceous plants like *Arabidopsis* and not sweet pepper, Moreover, DEGs possessing orthologues in pepper (718 up- and 405 down-regulated) and in *Arabidopsis* (393 up- and 324 down-regulated), that are assumed to have similar or identical functions in both plants, are differentially expressed in only one species. Likewise, the DEGs not possessing orthologues, in pepper (1079 up- and 533 down-regulated) and in *Arabidopsis* (1168 up- and 576 down-regulated), are also species-specifically differentially expressed. The species-specific differential expression of orthologues possessing and non-orthologue possessing DEGs explicitly indicates that the majority of the transcriptome response of *Arabidopsis* and pepper to WFT feeding is plant species-specific. Broadly, similar observations were reported to each subset of DEGs between *Arabidopsis* and tomato microarray-based transcriptome data generated in response to another cell-content feeder, the spider-mite *T. urticae* (Martel *et al*., 2015). Taken together, JA is found to be a prominently conserved pathway in *Arabidopsis* and pepper responses to WFT feeding, with the majority of the transcriptome response being species-specific.

Conclusion

In conclusion, this study provides high-resolution information on the temporal transcriptomic response of sweet pepper to WFT feeding. Through in-depth bioinformatic analysis, this study captured the temporal transcriptional reprogramming and its regulators and chronology of underlying defence mechanisms. Moreover, a comparison with the WFT-induced *Arabidopsis* transcriptome shows more commonalities in induced responses, with a prominent involvement of JA biosynthesis and signalling, than for suppressed responses. Thus, this detailed, in-depth *in-silico* analysis provides important insights into the dynamic and complex response of pepper plants to infestation with a cell-content feeding herbivorous insect.

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Supplementary figure

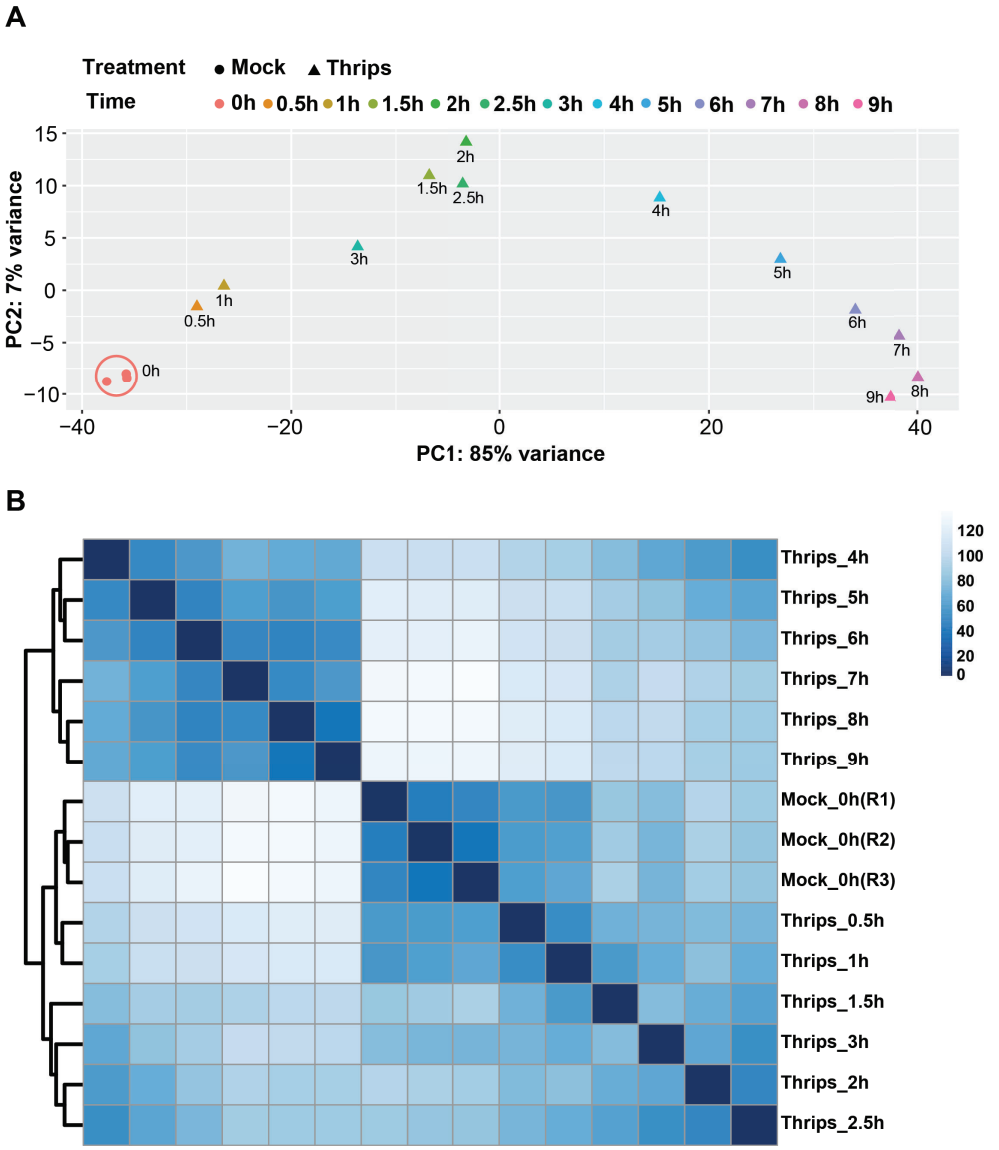

Figure S1. PCA and sample-to-sample distance analysis of transcriptome of sweet pepper infested (thrips) and non-infested (control/mock) plants. (A) PCA plot of sweet pepper non-infested (mock) and infested (thrips) transcriptome. PCA was generated on the regularized log_2-transformed data with DESeq2 package in R. Colours and shape indicate time points and treatments, respectively. Variation in percentage within the samples is depicted on both axes. **(B)** Sample-to-sample plot of sweet pepper non-infested (mock) and infested (thrips) transcriptome. It was generated on the regularized log_2-transformed data with DESeq2 R package.

Chapter 5

Comparative high-density transcriptomics reveals rapid and complex rearrangement of white cabbage transcriptome in response to thrips feeding

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Abstract

Temporal transcriptional rearrangements in response to insect feeding underlie a dynamically changing plant phenotype. Transcriptional rearrangements in response to the same herbivore in different plant species share commonalities but also have specific elements. To elucidate these, comparative transcriptomics using a high-density RNA-Seq approach provides a valuable tool. Here, we made a high-density (7 time points within 8 h) gene expression analysis of white cabbage leaves in response to onion thrips (*Thrips tabaci*) feeding. One tenth (3790 up- and 2009 downregulated) of the white cabbage genome is differentially expressed within 8 h of onion thrips feeding. Cluster analysis identified 48 co-expressed gene clusters (32 up- and 16 downregulated) of which the up- and downregulated clusters are broadly associated with defence and development-related GO functional categories, respectively. Genes associated with phytohormones (JA, ET and SA) and secondary metabolites (phenylpropanoids, flavonoids, green-leaf volatiles and indolic glucosinolates) were rapidly induced, whereas the aliphatic glucosinolate pathway and development-related processes were suppressed. Comparative analyses between the onion-thrips induced transcriptome of white cabbage and the Western flower thrips (WFT)-induced transcriptome of *Arabidopsis* and sweet pepper revealed that the majority of the full-genome transcriptional responses against thrips are system-specific. More commonalities were found among upregulated genes than among downregulated genes. This suggests that the activation of biological processes is more similar among plants than the deactivation of biological processes. TF families like MYB, bHLH and WRKY were conserved in regulating responses to thrips across three plant species. A prominently conserved element among the three plant species is the JA biosynthesis and signalling pathway. The response of white cabbage to onion thrips is faster than the response of *Arabidopsis* and sweet pepper to WFT. This includes genes involved in the biosynthesis of phytohormones and secondary metabolites. This high-density comparative transcriptomic analysis provides insight into the complexity of the temporally dynamic response of plants to feeding by cell-content-feeding thrips.

Keywords: white cabbage (*Brassica oleracea*), onion thrips, time series, RNA-Seq, high-resolution, comparative transcriptomics, herbivory, defence

Introduction

Plants face the attack by a wide range of herbivorous insects. The attack of a plant by an insect activates various mechanisms on a timescale of seconds to days. Upon herbivore perception, the earliest response is a change in plasma membrane potential (V_m) involving fluctuations of cytosolic Ca^{2+} concentrations and followed by production of H_2O_2 (Maffei *et al.*, 2007). Subsequently, kinases and phytohormones are induced. The phytohormones jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) are central players in regulating defences against different herbivorous insects (Pieterse *et al.*, 2009; Verhage *et al.*, 2010; Pieterse *et al.*, 2012; Stam *et al.*, 2014). For example, the JA signalling pathway is induced by chewing insects like caterpillars (Reymond *et al.*, 2004; De Vos *et al.*, 2005) and cell-content-feeding insects like thrips (Abe *et al.*, 2008; Abe *et al.*, 2012; Sarde *et al.*, 2018a; Steenbergen *et al.*, 2018), whereas the SA signalling pathway is induced by phloem-feeding insects like aphids and whiteflies (Zhu-Salzman *et al.*, 2004; Walling, 2008; Pieterse *et al.*, 2012; Tzin *et al.*, 2015; Broekgaarden *et al.*, 2018). Moreover, ethylene (ET) often synergises with JA and fine-tunes JA-regulated defences against herbivorous insects (Pieterse *et al.*, 2009; Pieterse *et al.*, 2012; Stam *et al.*, 2014). Phytohormones regulate the activation of gene transcription (time scale of minutes to hours) and the biosynthesis of metabolites (time scale of hours to days) (Maffei *et al.*, 2007; Stam *et al.*, 2014).

Thus, herbivore attack results in a dynamic reconfiguration of their transcriptome. Such transcriptional rearrangements result in a temporally dynamic reorganization of various biological processes (Windram *et al.*, 2012; Lewis *et al.*, 2015; Hickman *et al.*, 2017; Sarde *et al.*, 2019; Steenbergen *et al.*, 2019). The temporal transcriptional rearrangements include the induction and repression of transcription factors (TFs) that regulate genes and processes involved in the biosynthesis of compounds such as phytohormones, primary and secondary metabolites, defence-related proteins or development-related pathways. This reorganization of transcriptional and biological processes dynamically influences the plant phenotype (Stam *et al.*, 2014), subsequently altering interactions of the plant with plant-associated organisms. This can influence plants throughout the season (Poelman *et al.*, 2010) or even over different seasons (Stam *et al.*, 2018). Thus, early transcriptional responses of plants to attack by herbivorous insects can have extensive impact on plant ecology.

Studies of transcriptional responses to insect feeding usually include one or two time points over a period of 24 hours or longer, thus providing a low-resolution representation of the interaction (De Vos *et al.*, 2005; Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011; Zhang *et al.*, 2013; Appel *et al.*, 2014; Diaz-Riquelme *et al.*, 2016; Kroes *et al.*, 2017; Broekgaarden *et al.*, 2018; Tu *et al.*, 2018). To gain comprehensive insight into how a plant temporally rearranges its transcriptome in response

to attack by an insect herbivore, the collection of early high-density time-series transcriptional data is crucial. Such data are instrumental for unravelling the early phase of defence responses to insect herbivores, including the involvement of major TFs, phytohormones, sequential activation of biological processes and gene regulatory networks.

Furthermore, because high-density transcriptional analyses provide detailed information on early events in a plant's response, the data can be used to compare different insect-plant interactions for e.g. speed of response, chronology of gene transcriptional processes and biological processes activated, and complexity of temporal gene expression patterns. Recently, we made a high-resolution temporal assessment of *Arabidopsis* (Steenbergen *et al*., 2019) and sweet pepper (*Capsicum annuum*) (Sarde *et al*., 2019) transcriptomes in response to western flower thrips (WFT, *Frankliniella occidentalis*) feeding. This was done by detailed analysis of the transcriptome for 12 and 7 time points within the first 8 hours of attack on *Arabidopsis* and sweet pepper plants, respectively. This has yielded extensive information on similarities and differences in how these plants of different families respond to thrips infestation. In the present study, we made a high-resolution analysis of a third plant-thrips interaction, i.e., between white cabbage (*Brassica oleracea*) and onion thrips. One of the foci of this study is to analyse the expression pattern of white-cabbage genes involved in the biosynthesis of the defensive secondary metabolites of brassicaceous plants, such as glucosinolates and flavonoids (Schoonhoven *et al*., 2005).

Onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae), is a serious pest worldwide on various crops including White cabbage (Shelton *et al*., 2008; Fail *et al*., 2013). The pest status of onion thrips can be attributed to several characteristics, such as its short life-cycle, high reproductive rate, polyphagous nature, thigmokinetic behaviour and rapid development of resistance to insecticides (Diaz-Montano *et al*., 2011; Gill *et al*., 2015; Steenbergen *et al*., 2018). These insects cause direct damage to plants by rupturing the epidermal and mesophyll cells and ingesting the cell contents. Moreover, they also cause indirect damage on plants by transmitting tospoviruses like Iris yellow spot virus (IYSV) (Bunyaviridae) (Diaz-Montano *et al*., 2011; Gill *et al*., 2015).

In contrast to WFT, molecular responses of plants to onion thrips have not received much attention. The main goals of the present study were: 1) to comprehensively investigate the temporal transcriptomic response of white cabbage to feeding by onion thrips through high-resolution transcriptomics, and 2) to compare the response of white cabbage plants to onion thrips to the transcriptomic response of *Arabidopsis* and sweet pepper plants to WFT through comparative transcriptomics to extend our knowledge of thrips-induced transcriptional plant responses.

Materials and methods

Plant material and onion thrips

White cabbage [*Brassica oleracea* (W0246 variety, Syngenta, Enkhuizen, The Netherlands)] plants were grown in a greenhouse at 22 ± 5°C (day/night), 16L:8D photoperiod, 60 ± 10% relative humidity and 130 µmol photons m^{-2} s^{-1} of light intensity. For thrips infestation, four-week-old white cabbage plants were used. Onion thrips (*Thrips tabaci*) were reared in jars (10 cm diameter) on leek leaves (*Allium ampeloprasum*) in a climate-controlled room (25 ± 2°C, L16:8D photoperiod and 70 ± 10% relative humidity).

Thrips treatment, RNA extraction and library preparation

Five 2nd instar larvae (L2) of onion thrips, confined in clip cages (3 cm diameter), were used to infest the second true leaf of four-week-old white cabbage plants. One clip cage was used per plant. Empty clip cages without thrips served as mock treatment. For each time point and treatment, the leaf area underneath the clip cages was harvested using a cork borer (3 cm diameter), flash frozen in liquid nitrogen and stored at -80 °C. Time points of harvesting were 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, and 9 h after the introduction of the five thrips larvae. Three biological replicates were collected for each time point and treatment. Each individual biological replicate represents one single white cabbage plant. Extraction of RNA was executed using the RNeasy Plant Mini Kit (QIAGEN), according to the manufacturer's protocol. DNAase I treatment on column was conducted for all the samples during RNA extraction. RNA quantity and quality were assessed by Nanodrop and Agilent 2100 bioanalyzer, respectively. Samples with RNA Integrity Number (RIN) ≥ 7 were used for RNA library preparation. Samples were prepared according to the TruSeq Stranded mRNA HT Sample Prep Kit from Illumina (Illumina Inc., San Diego, CA, USA). This protocol identifies strand-specific transcripts. Sequencing of samples was performed with an Illumina Hi-seq 2000 platform. Samples were randomly allocated to seven lanes (Illumina flow cells) within each run.

Quality control, alignment and differential gene expression of RNA-Seq dataset

Quality of raw RNA-Seq reads was assessed using the FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) tool. Trimmomatic was used to trim the initial 14-basepairs (bp) and Illumina adapters. Reads below 25 bp length were excluded from analysis for all samples (Bolger *et al.*, 2014).

Alignment of RNA-Seq reads was performed using TopHat2 (v2.0.14) (Kim *et al.*, 2013) using the following parameters: 'p 4', '--bowtie-n', '--min-intron-length 40', '--max-intron-length 2000', '-N 4', '--no-novel-juncs', '--read-gap-length 2', '--read-edit-

dist 4'. The aligned reads to each *B. oleracea* (v2.1) (Parkin *et al.*, 2014) gene model were summarized using HTSeq-count (v.0.9.1) (Anders *et al.*, 2015) with parameters: '--stranded no', '-i ID', '–t mRNA'. Principal Component Analysis (PCA) and sample-to-sample distance plots were generated in R (https://www.r-project.org/) using regularized $\log_2$-transformed data using the DESeq2 package (Love *et al.*, 2014; Love *et al.*, 2015).

For analysis of differentially expressed genes (DEG), the DESeq2 R Bioconductor package (Love *et al.*, 2014; Love *et al.*, 2015) in R was used. Prior to DEG analysis, raw read counts were normalized for sequencing depths across all samples using the DESeq2's count normalization procedure. A negative binomial likelihood ratio test (nbinomLRT) was used to identify DEGs between mock and thrips-infested plants. For this analysis, both treatment and time post-treatment were considered as factors. Genes with $\log_2$-fold change ≥ 0.5 or ≤ - 0.5 at one or more time points, a Bonferroni-corrected *P* value < 0.01 and read counts ≥ 20 at least in one sample were qualified as DEGs.

Gene clustering, TF family and promoter motif enrichment analyses

SplineCluster, a time series-clustering algorithm, was used on $\log_2$-FC profiles of DEGs at each time point, to partition the DEGs into clusters based on temporal expression profiles (Heard *et al.*, 2006). SplineCluster was used with the following parameters: a prior precision stringency of 10^{-4}, the default normalization procedure and cluster reallocation step (Heard, 2011). Default values were used for all other optional parameters.

For TF family abundance analysis, we investigated overrepresentation of TF families within DEGs induced upon thrips feeding in white cabbage. Before this analysis, the TF families in white cabbage were determined using 4272 TFs of *B. oleracea* from the Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/index.php) (Jin *et al.*, 2017) using blastp with stringency E-value < 10^{-4}. The hypergeometric distribution was used to identify overrepresentation of TF families within sets of DEGs. *P* values were corrected for multiple testing with the Bonferroni method.

To identify TF binding motifs in promoters of temporally clustered DEGs, comparison was made to 580 characterized *Arabidopsis* TF DNA-binding motifs from Franco-Zorrilla *et al.* (2014) and CIS-DB (version 1.02) (Weirauch *et al.*, 2014). FIMO (Grant *et al.*, 2011) was used to determine the occurrence of particular motifs within the promoter sequences (500 bp upstream to the start codon) of all white cabbage genes. A motif was considered to be present in a promoter sequence, if it had at least one match with a P value < 10^{-4}. The hypergeometric distribution against the background of all white cabbage genes was used to assess motif enrichment in a given cluster.

Identification of chronology of defence pathways upon thrips feeding

Pairwise comparison between mock and thrips-treated samples at each time point was performed using DESeq2 package (Love *et al.*, 2014; Love *et al.*, 2015) in R. Genes with $\log_2$-fold change ≥ 0.5 or ≤ - 0.5 and a Bonferroni-corrected *P* value < 0.01 were considered to be differentially expressed. For the small number of genes that did not meet these criteria, the time point of first differential expression was defined by minimal *P* value. The output files were further processed to identify and categorize DEGs into two categories: **f**irst **t**ime **o**f **d**ifferential **e**xpression (ftode) and **a**gain **d**ifferential **e**xpression (ade).

Gene Ontology (GO)-term enrichment analysis

For GO-term enrichment analysis, Cytoscape (Shannon *et al.*, 2003) was used for *Arabidopsis* and GOAtools (v0.7.9) (Klopfenstein *et al.*, 2018), a python-based library, was used for white-cabbage and sweet pepper, using Fisher's exact test. For this, we used the GO-annotated proteome for *B. oleracea* from the Plaza database (https://bioinformatics.psb.ugent.be/plaza/) (Jin *et al.*, 2017). Overrepresentation of GO categories like "biological process", "cellular component" and "molecular function" was analyzed at $P < 0.05$.

Comparative transcriptomics

Comparative transcriptomics was performed between *B. oleracea* and *Arabidopsis* (TAIR10) and between *B. oleracea* and *Capsicum annuum L.* Zunla proteome using the **B**i-directional **B**est **H**it (BBH) method (Martel *et al.*, 2015). Local blastp with stringency E-value < 10^{-4} was used to identify one-to-one orthologues in both comparisons. The *Arabidopsis* and pepper transcriptome datasets used in this study relate to plants induced by *Frankliniella occidentalis* (Western flower thrips) according to methodology very similar to that used in the present study (Sarde *et al.*, 2019; Steenbergen *et al.*, 2019).

Results

Temporal transcriptomic response of white cabbage plants upon onion thrips feeding

To gain detailed insight into the temporally dynamic transcriptomic response of white cabbage plants upon feeding by onion thrips, individual samples from 12 time points (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 h of infestation) plus 3 samples of the 0 h time point were sequenced and analysed. The PCA and heat map of sample-to-sample distance analysis show a gradual development of the transcriptom-

ic response with time in the white cabbage plants (Fig. S1). This analysis led to the selection of the 1, 2, 3, 4, 6 and 8 h time points for sequencing of additional samples and subsequent in-depth analysis. Thus, including the 0 h time point, the time series consists of seven time points. The detailed data for these seven time points were subjected to PCA to investigate the effect of treatment and time on the white cabbage transcriptome in response to onion thrips feeding. PCA explicitly discriminates the samples for treatments and time points, indicating that both treatment and time since infestation are determinants of the transcriptomic response of white cabbage (Fig. 1A). The mock-treated samples differ between time points, suggesting a diurnal-rhythm effect on the transcriptome. The three replicates per treatment and time point exhibit considerable similarity apart from the first time point for thrips-infested plants (1h).

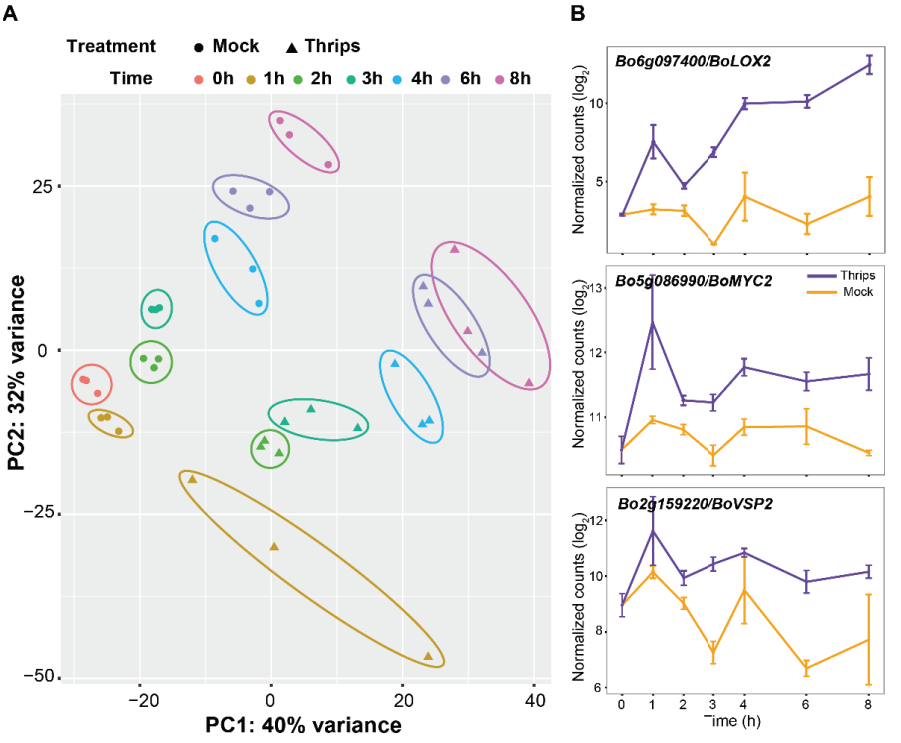

Figure 1. Principal component analysis (PCA) of white-cabbage transcriptome of mock-treated and onion thrips-infested plants at several time points and expression patterns of selected JA-associated marker genes of white cabbage. (A) PCA plot of white-cabbage whole-genome transcriptomic response at seven time points for mock-treated and thrips-infested plants. Variation between the samples of treatment and time post-treatment is depicted on both axes. Different shapes represent the different treatments and the different colours represent the different time points. (B) Temporal expression profile of JA-related marker genes from transcriptome dataset. Expression of each gene depicts mean ± SE of 3 biological replicates.

Next, we used the DESeq2 negative binomial log-ratio test with treatment and time since start of treatment as factors, to identify 5799 differentially expressed genes (DEGs) between mock (Supplemental Data Set 1). This accounts for 9.7 % of the total 59,225 white cabbage genes (Parkin *et al.*, 2014). Because thrips are known to induce the JA pathway (De Vos *et al.*, 2005; Abe *et al.*, 2008; Sarde *et al.*, 2018a; Sarde *et al.*, 2018b), the expression profiles of several genes in the JA pathway (*BoLOX2*, *BoMYC2*, *BoVSP2*) were specifically analysed. Reassuringly, all three genes were rapidly up-regulated by thrips feeding (Fig. 1B).

Gene clusters, TF family abundance and TF motif analysis

To identify the predominant dynamic patterns of gene expression in plants during thrips feeding, we used the time-series-clustering algorithm, SplineCluster (Heard *et al.*, 2006), to cluster the 5799 DEGs based on their expression pattern over time. The SplineCluster analysis identified 48 coexpressed gene clusters: 16 down-and 32 upregulated clusters. The 16 downregulated (clusters 1-16) and 32 upregulated (clusters 17-48) gene clusters represent 2009 (34.6%) and 3790 (65.4%) DEGs, respectively (Fig. 2A) (Supplemental Data Set 2). Among the upregulated clusters, several (clusters 20-29, 35 and 41-48) showed that gene expression is rapidly induced within 1 h of thrips feeding, whereas in other clusters (cluster 30-34 and 36-40) genes were initially downregulated, followed by subsequent upregulation. Similarly, in the clusters representing downregulated genes, rapid downregulation is seen in some clusters (4-10), while genes in other clusters (1, 2 and 11-16) show a gradual downregulation over time (Fig. 2A). To explore the biological processes associated with the dynamic expression patterns identified by the SplineCluster analysis, GOATOOLS (Klopfenstein *et al.*, 2018) was used to identify significantly overrepresented functional categories associated with genes in each cluster. Several upregulated clusters (clusters 42-45 and 48) are enriched with genes associated with GO terms like “Jasmonic acid signalling pathway” and “Response to JA” (Fig 2A). Other defence-related pathways are overrepresented in other clusters. For example, cluster 27 with “Tryptophan biosynthetic process”, cluster 36 with “Anthocyanin-containing compound biosynthesis”, cluster 35 with “Defence response to fungus” (Supplemental Data Set 3). Similarly, downregulated clusters (clusters 1-16) exhibit GO terms especially associated with growth and development (Supplemental Data Set 4). For example, clusters 2 and 3 with “Photosynthetic electron transport in photosystem I”, cluster 4 with “Photosynthesis, light harvesting”, clusters 12, 13 and 14 with “Protein phosphorylation” and cluster 16 with “Protein ubiquitination”. Cluster 12 is overrepresented with genes involved in “Terpenoid biosynthesis”.

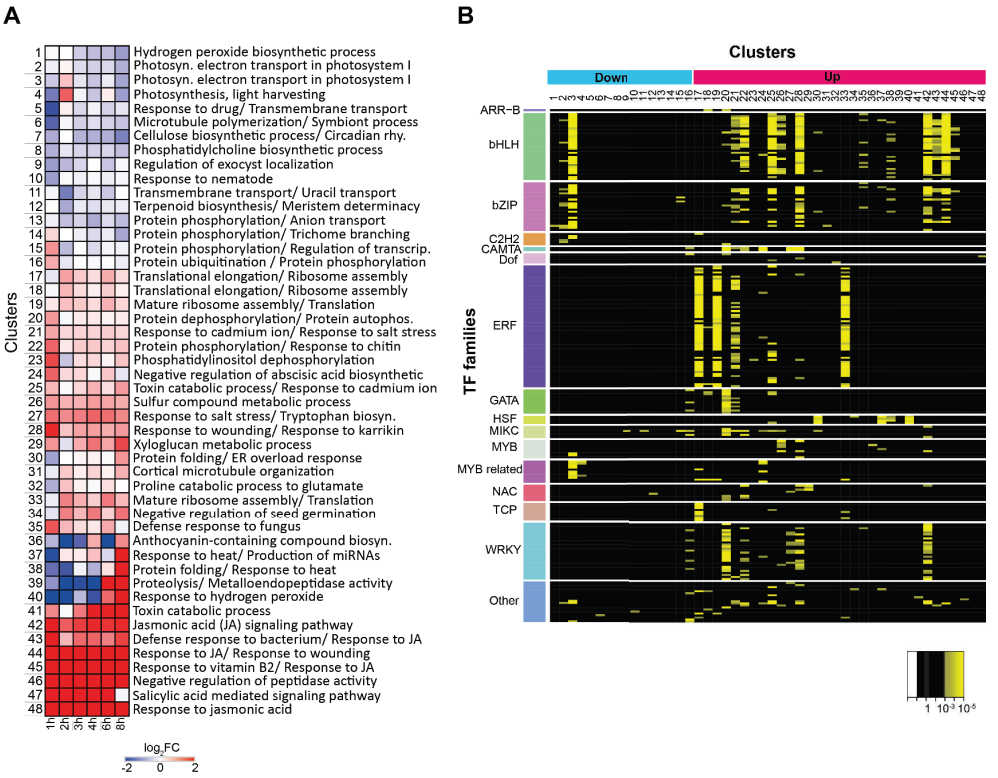

Figure 2. **Temporally co-expressed gene clusters and promoter motif analysis of gene clusters. (A)** Heat map showing mean expression profiles of 48 gene clusters (clusters 1-16 downregulated and clusters 17-48 upregulated) with selected GO terms. **(B)** Overrepresented TF binding motifs in the 48 gene clusters. Enriched TF family binding motifs in each cluster are shown in yellow colour.

5

To investigate which TF families are involved in regulating the white cabbage response to onion thrips feeding, TF abundance was analysed for all up- (3790) and downregulated (2009) genes. We identified 2910 non-redundant TFs in the *B. oleracea* (TO1434) proteome (Parkin *et al.*, 2014) using 4272 TFs of *Brassica oleracea* from the Plant Transcription Factor Database (Supplemental Data Set 5). In the 3790 upregulated genes, several TF families like ERF, WRKY, MYB, HSF, GRAS, bHLH, MYB-related, RAV and Trihelix are overrepresented. Similarly, in the 2009 downregulated genes, the TF families ARF, NF-YA, YABBY, C2H2, MYB_related, CAMTA, AP2, G2-like, Nin-like, HD-ZIP, bHLH, TALE and Dof are significantly overrepresented (Supplemental Data Set 6).

Subsequently, we searched for TF binding motifs in the promoter sequences of the genes in all 48 clusters. For this, we used FIMO (Grant *et al.*, 2011) and 580 characterized *Arabidopsis* TF DNA-binding motifs from CIS-DB (version 1.02) (Franco-Zorrilla

et al., 2014; Weirauch *et al.*, 2014). In clusters of upregulated genes (clusters 17-48), motifs that correspond to bHLH, bZIP, ERF and WRKY TFs are overrepresented (Fig. 2B), suggesting expression of genes in these clusters is regulated by members of these TF families. The motif enrichment also matches the coordinated up-regulation of genes encoding members of these TF families following thrips feeding.. Clusters 22, 25, 28, 42 and 44 are enriched with binding sites of bHLH and bZIP TFs, clusters 17, 19 and 33 with binding sites of ERF TFs and clusters 20, 28 and 42 with binding sites of WRKY TFs. In clusters with downregulated genes (clusters 1- 16), binding sites for bHLH, bZIP, MYB-related and MIKC TFs are overrepresented (Fig. 2B).

Chronology of phytohormone induction

The phytohormones JA, ET and SA regulate induced defences against insect herbivores. To investigate how these phytohormonal pathways are involved in the response of white cabbage plants to onion thrips, we examined the temporal expression dynamics of genes involved in these pathways. Several of the JA-biosynthetic (*BoLOX2*, *BoAOS*, *BoAOC3*, *BoOPR3*, *BoACX1*) and JA-signalling (*BoJAZ1-3, BoJAZ5-9*) genes are significantly upregulated within 1h of thrips feeding (Fig. 3A). Downstream JA-responsive genes like *BoMYC2* and *BoVSP2* are significantly induced at 2 h and 3 h since the start of thrips feeding, respectively. Similarly, many ET-related genes are induced within 1-2 h of thrips feeding (Fig. 3B). SA biosynthesis may occur via the phenylalanine (PAL) or isochorismate pathway (ICS). Yet, the ICS pathway is crucial for the production of SA that is involved in plant defence against pathogens (Wildermuth *et al.*, 2001). We analysed the transcriptional responses of *PAL* and *ICS* genes. Several homologs of *PAL* genes are upregulated within 2h of thrips feeding. In contrast, *BoICS* is downregulated at the 3h time point. The downstream SA-responsive gene, *BoPR5* (Ali *et al.*, 2017), is significantly induced for the first time at the 6 h time point. This indicates downregulation of SA via the ICS pathway, upregulation of the phenylalanine (PAL) pathway and late upregulation of SA-responsive genes (Fig. 3C). In conclusion, this analysis suggests an induction of all three major defence-related hormones in white cabbage upon thrips feeding, albeit at different time scales.

Induction of secondary metabolites

To evaluate if feeding by onion thrips induces secondary metabolites such as phenylpropanoids, flavonoids, green leaf volatiles (GLVs) and glucosinolates (GLS), the temporal expression pattern of genes involved in these pathways was analysed. The majority of phenylpropanoid and flavonoid biosynthetic genes (*BoPAL*, *BoC4H*, *Bo4CL*, *BoF3H*, *BoFLS*) (Onkokesung *et al.*, 2014) show significant upregulation at the 2 h time point, with the exception of *BoCHS*, *BoCHI* and a few homologs of *Bo4CL*, which are downregulated at this time point (Fig. 4A).

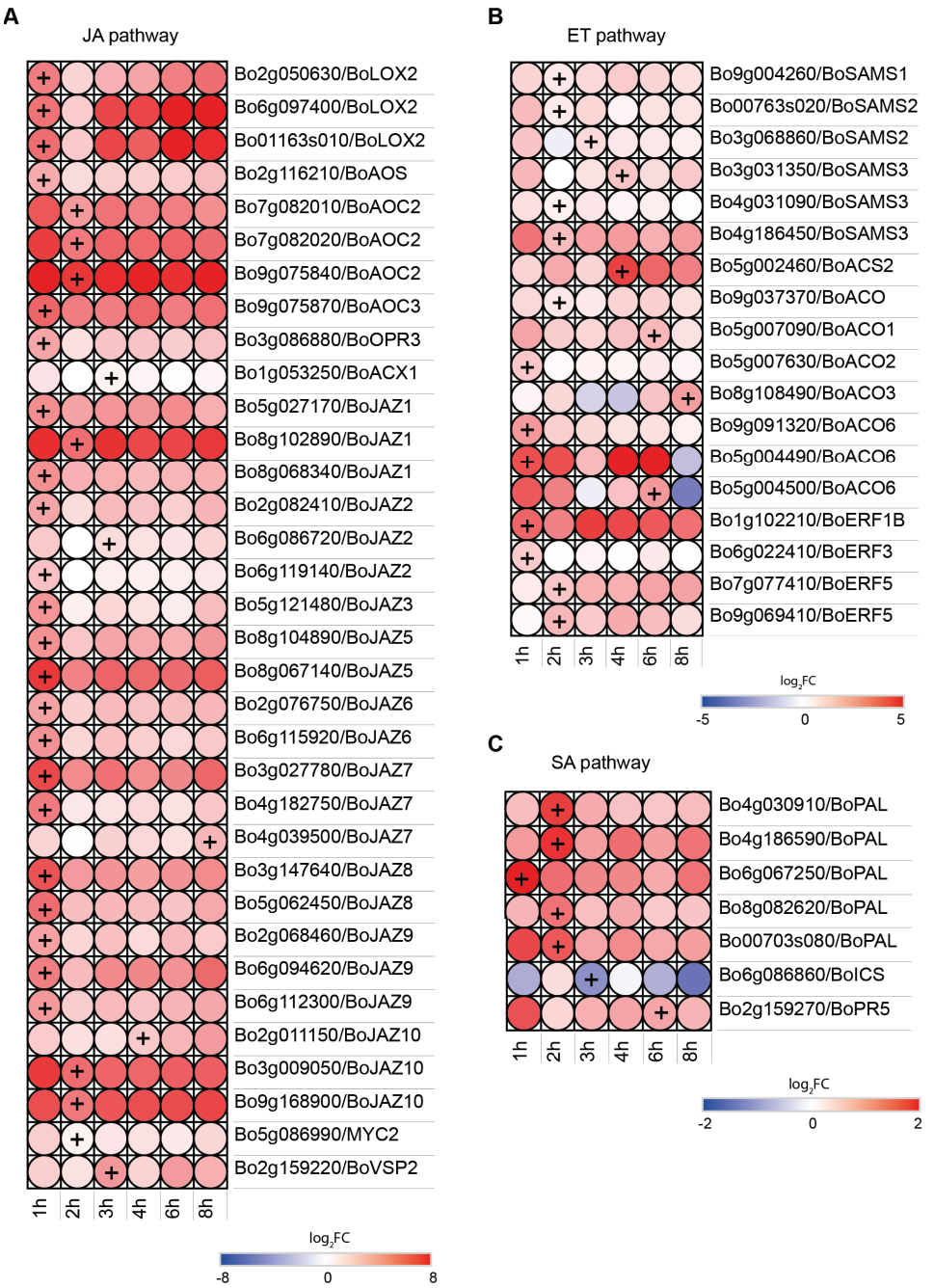

Figure 3. Temporal expression of genes involved in JA, ET and SA hormonal pathways differentially expressed upon onion thrips feeding. (A) JA pathway, **(B)** ET pathway and **(C)** SA pathway. Expression of genes represents $\log_2$ fold change between mock- and thrips-infested plants for each time point. '**+**' indicates significant ($P < 0.01$) first time of differential expression (ftode) for each gene.

Glucosinolates (GLS), are broadly divided into three groups: aliphatic, aromatic and indolic glucosinolates (Halkier & Gershenzon, 2006; Ishida *et al.*, 2014; Frerigmann *et al.*, 2016). Among the DEGs in our dataset, we found major regulators of indolic glucosinolates (*BoMYB34*, *BoMYB122*, *BoMYB51*) (Frerigmann *et al.*, 2016) and several downstream genes (*BoASA1*, *BoTSA1*, *BoCYP83B1, BoGSTF9*, *BoGSTF10*, *BoSUR1, BoUGT74B1, BoSOT16*) (Tytgat *et al.*, 2013) to be induced within 1-2 h of thrips feeding (Fig. 4B). In contrast, the major upstream regulator of aliphatic GLS (*BoMYB28*) (Halkier & Gershenzon, 2006) was suppressed within 2 h of thrips feeding (Fig. 4C). In addition, the expression of various other major genes involved in the biosynthesis of aliphatic GLS, such as *BoMYB29*, *BoMYB76*, *BoBAT5* and *BoMAM1-3* (Tytgat *et al.*, 2013) did not alter upon thrips feeding. This suggests that specific induction of indolic GLS and suppression of aliphatic GLS occurs upon onion thrips feeding. Both homologues of the hydroperoxide lyase (*HPL*) gene, known to break down lipid hydroperoxides to produce green leaf volatiles (Bate *et al.*, 1998), are upregulated within 1 h of thrips feeding (Fig. 4D).

Chronology of biological processes altered in response to thrips feeding

To gain in-depth understanding of how white cabbage plants reconfigure their transcriptome over time and sequentially activate different biological processes, we performed a pairwise comparison for all individual time points between mock treatment and thrips treatment. This analysis showed progressive transcriptional reconfiguration over time and partitioned both up- and downregulated DEGs into the classes **f**irst **t**ime **o**f **d**ifferential **e**xpression (ftode) and **a**gain **d**ifferentially **e**xpressed (ade) (Supplemental Data Set 7). The major transcriptional burst in both up- and downregulated genes, occurred rapidly, i.e. within 1-2 h of thrips feeding (Fig. 5). The majority of DEGs in this study are differentially expressed within 2 h since the start of thrips feeding. Approximately 650-800 of the upregulated genes at 3-8 h were also upregulated at earlier time points. In contrast, only few downregulated genes at 3-8 h since the start of thrips feeding are again upregulated, suggesting that the upregulation of genes is more pronounced than the downregulation. Thus, this analysis illustrates temporal reconfiguration of the white-cabbage transcriptome and its initial transcriptional burst in response to thrips feeding occurring within 1-2 h of thrips feeding.

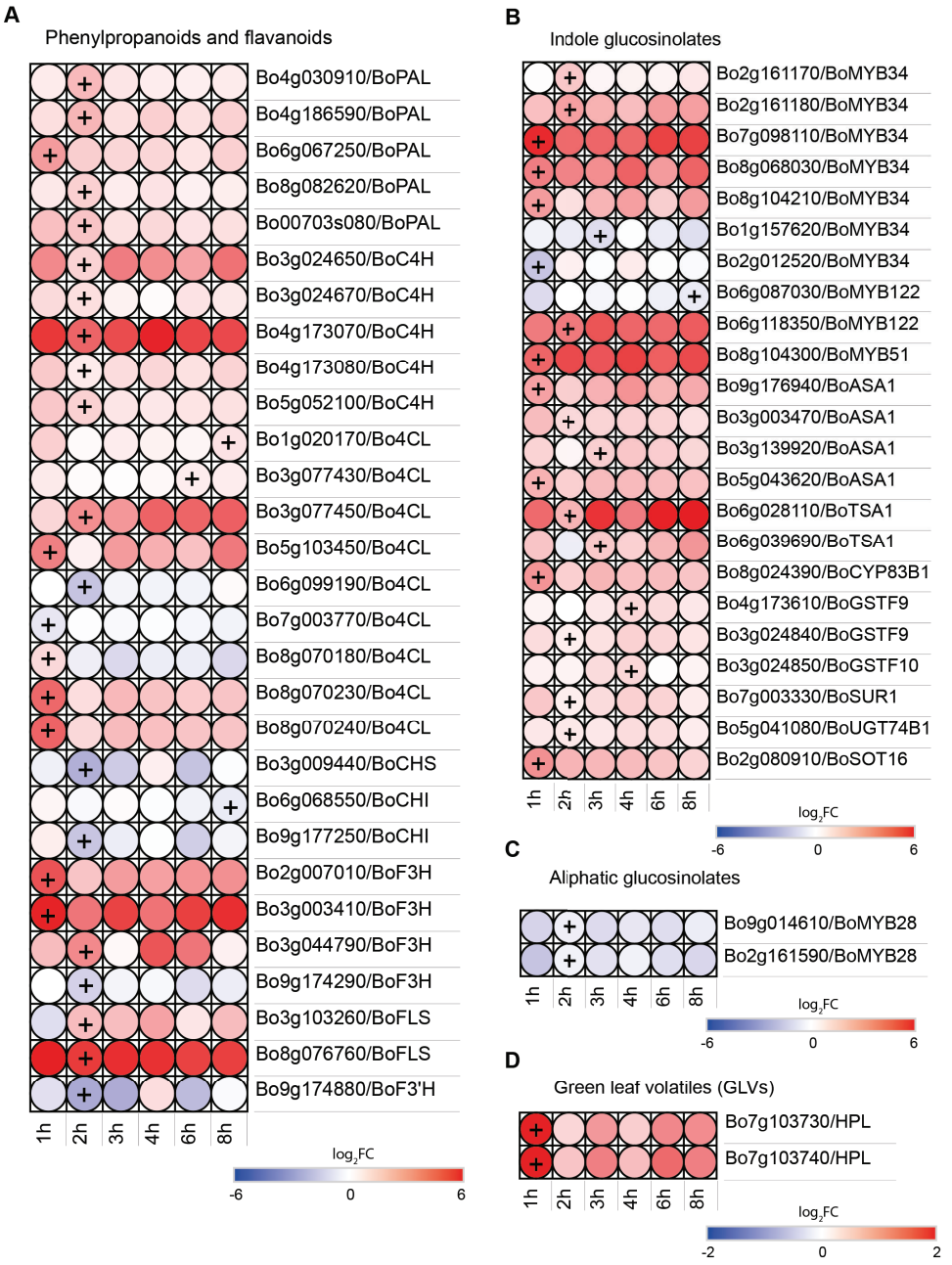

Figure 4. Temporal expression of genes involved in the biosynthesis of secondary metabolites . (A) phenylpropanoid and flavonoid pathways, **(B)** Indole glucosinolates (GLS), **(C)** Aliphatic glucosinolates (GLS), and **(D)** green-leaf volatiles. Expression of genes represents log_2 fold change between mock- and thrips-infested plants for each time point. '**+**' indicates significant ($P < 0.01$) first time of differential expression (ftode) for each gene.

To analyse the chronology of biological processes activated upon thrips feeding, we investigated the GO terms associated with genes showing first time of differential expression (ftode) at each time point. At 1h after the start of thrips feeding, the GO terms overrepresented among upregulated genes are mainly associated with functional categories like “Jasmonic acid mediated signalling pathway” and “Response to ethylene”, indicating rapid induction of these phytohormonal pathways (Fig. 5). At the 2 h time point, GO terms like “Regulation of systemic acquired resistance”, “Aromatic amino acid family biosynthetic process” are overrepresented, reflecting induction of resistance and biosynthesis of aromatic amino acids (Phe, Tyr, Trp), which act as precursors for several secondary metabolites in plants (Tzin & Galili, 2010). Among the downregulated genes, GO terms associated with plant development like “Response to auxin”, “Photosynthesis”, and “Photosynthetic electron transport in photosystem-I” are overrepresented.

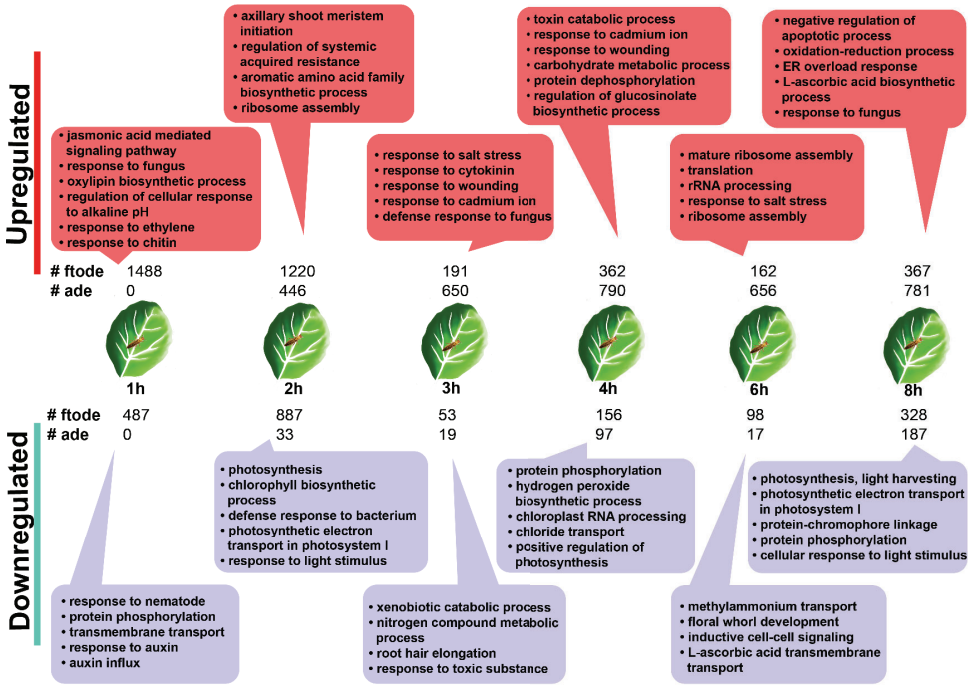

Figure 5. Chronology of white cabbage biological processes reorganized in response to onion thrips feeding. Number of first time differentially expressed (ftode) and again differentially expressed (ade) genes for both up- and downregulated genes are depicted above and below pictures of cabbage leaves, respectively. Light green and light blue boxes show selected GO terms for ftode up- and downregulated genes, respectively.

Comparative transcriptomics: white cabbage vs *Arabidopsis* and white cabbage vs sweet pepper

Recently, similar high-density time series data were generated for *Arabidopsis* (Steenbergen *et al*., 2019) and sweet pepper (*Capsicum annuum*) (Sarde *et al*., 2019) plants in response to WFT feeding. These studies identified a total of 2788 (1820 up- and 968 downregulated) DEGs in *Arabidopsis* and 3062 (2060 up- and 1002 downregulated) DEGs in sweet pepper over a span of 8 hours of thrips feeding. To gain insight into the commonalities and specifics of whole-genome transcriptional responses on plant-family and plant-species level upon feeding by different thrips species, we compared onion thrips induced white-cabbage DEGs (5799; 3790 up- and 2009 downregulated) with WFT-induced *Arabidopsis* (Steenbergen *et al*., 2019) and sweet pepper DEGs (Sarde *et al*., 2019).

In the comparison of DEGs between white cabbage and *Arabidopsis,* 1938 of 3790 upregulated genes and 1093 of 2009 downregulated genes from white cabbage have *Arabidopsis* orthologues. Likewise, 1420 of 1820 upregulated genes and 708 of 968 downregulated genes from *Arabidopsis* have white cabbage orthologues (Fig. 6). In the comparison of white cabbage vs sweet pepper, we identified that 1126 of 3790 upregulated genes and 730 of 2009 downregulated genes of white cabbage possess sweet pepper orthologues. Likewise, 986 of 2060 upregulated genes and 468 of 1002 downregulated genes of sweet pepper possess white cabbage orthologues (Fig. 7). The relatively high number of orthologues between white cabbage and *Arabidopsis* compared to the comparison of white cabbage and sweet pepper, reflects their shared brassicaceous identity. Furthermore, in both comparisons, large numbersof genes [in white cabbage vs *Arabidopsis*, 1225 up- and 970 downregulated in white cabbage and 754 up- and 538 downregulated in *Arabidopsis*; in white cabbage vs sweet pepper, 779 up- and 602 downregulated in white cabbage and 624 up- and 355 downregulated in sweet pepper] have orthologues in the other plant species, but those orthologues are not differentially expressed. Thus, a large proportion of DEGs show differential expression in a species-specific manner (Fig. 6 and 7). This indicates that the majority of the transcriptomic responses in each plant species is distinct. It is interesting to note that more commonalities exist in the subset of upregulated genes than in the subset of downregulated genes: 622 of the 1938 (32%) upregulated white cabbage genes that have *Arabidopsis* orthologues are also upregulated in *Arabidopsis*, compared to 79 out of 1093 (7%) downregulated white cabbage genes with orthologous *Arabidopsis* genes that are downregulated in both species. Moreover, this is similar for the white cabbage vs sweet pepper comparison: 315 upregulated genes out of 1126 (27%) differentially regulated sweet pepper genes that are upregulated in sweet pepper and cabbage versus 81 out of 730 (11%) downregulated genes. This suggests that the activation of biological processes is more similar among plants than

the deactivation of biological processes.

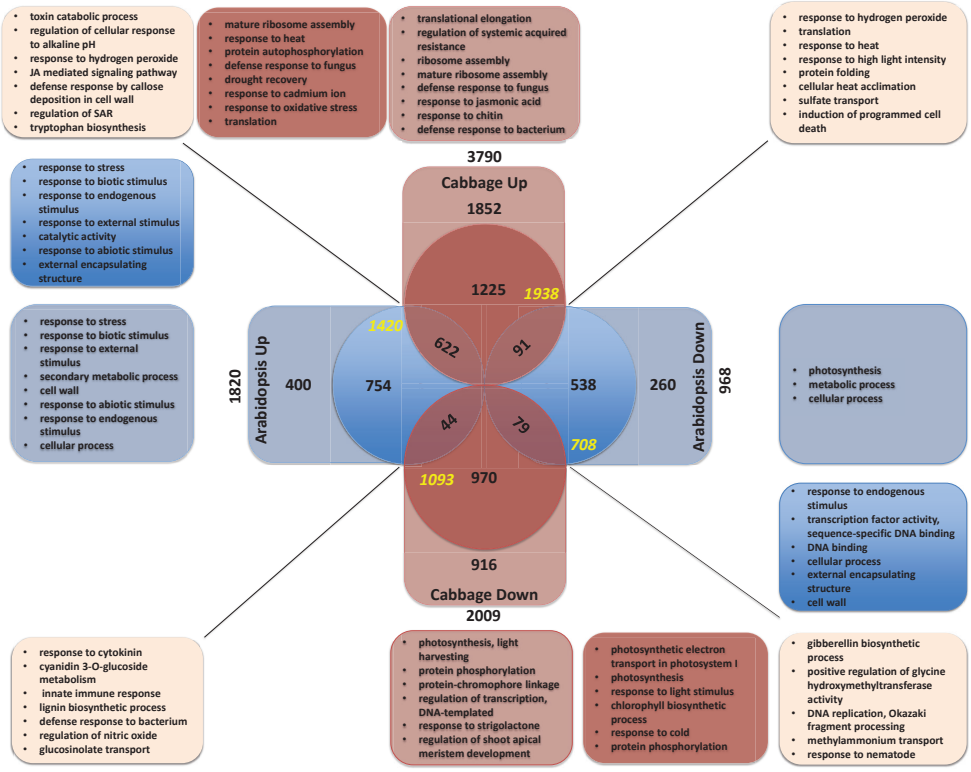

Figure 6. Venn diagram depicting the numbers of genes and biological processes that are similarly or differentially regulated during feeding by western flower thrips on *Arabidopsis* and by onion thrips feeding on white-cabbage plants. Selected overrepresented GO terms are depicted for each subset of genes. Brown and blue colours represent white cabbage and *Arabidopsis* DEGs, respectively. Dark blue and dark brown segments represent genes with orthologues in the other plant species, light blue and light brown represent genes without orthologues in the other plant species. Yellow numbers depict total numbers of genes with orthologues in the other plant species for each subset of genes.

GO term analysis for each subset of genes was performed for both comparisons (white cabbage vs *Arabidopsis* and white cabbage vs sweet pepper) (Fig. 6 and 7). Common upregulated genes (622 in white cabbage *vs Arabidopsis* and 315 in white cabbage *vs* sweet pepper) from both comparisons are associated with JA-related GO terms, such as “JA mediated signalling pathway” and “Response to JA”. This exhibits the conservation and importance of the JA pathway in all three plant species against thrips feeding. Moreover, in the white cabbage vs sweet pepper comparison, the sweet pepper DEGs (624) with orthologues in white cabbage, that are species-specifically induced in sweet pepper are associated with the GO term “Isoprenoid (terpenoid) biosynthesis”, indicating that this pathway is especially important in sweet

pepper. In downregulated genes for both comparisons, GO terms are largely associated with development-related processes, such “Photosynthesis”, “Photosynthetic electron transport in photosystem I”, “Photosynthesis, light harvest”, “Chlorophyll biosynthetic process” (Fig. 6 and 7). This indicates that downregulation of developmental processes occurs in all three plant-thrips interactions.

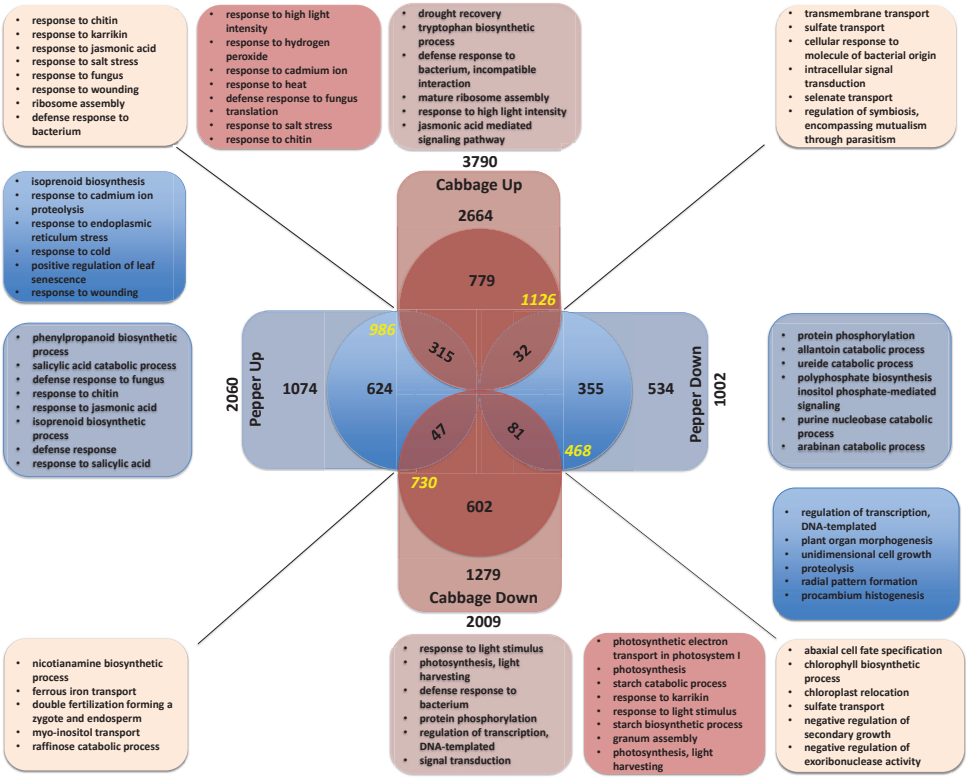

Figure 7. **Venn diagram depicting the numbers of genes and biological processes that are similarly or differentially regulated during feeding by western flower thrips on sweet pepper plants and by onion thrips feeding on white cabbage plants**. Selected overrepresented GO terms are depicted for each subset of genes. Dark blue and dark brown segments represent genes with orthologues in the other plant species, light blue and light brown represent genes without orthologues in the other plant species. Yellow numbers depict total numbers of genes with orthologues in the other plant species for each subset of genes.

Discussion

In the present study, we aimed to gain comprehensive insights into the temporally dynamic full-genomic transcriptional response of white cabbage plants to feeding by onion thrips and to elucidate the level of conservation in induced defences in different plants, induced by two thrips species. We recorded that 9.7% (5799/59225) of the

white cabbage genes is differentially expressed within 8 h of onion thrips feeding and that the transcriptional patterns of these 5799 genes can be categorized into 48 co-expressed gene clusters (32 up- and 16 downregulated). The up- and downregulated gene clusters are broadly associated with defence and development-related biological processes, respectively. The majority of transcriptional reprogramming occurs already within 2 h after the start of exposure to thrips. Processes induced include the biosynthesis of phytohormones (JA, ET and SA) and secondary metabolites (phenylpropanoids, flavonoids, GLVs and indolic GLS), whereas developmental processes and the aliphatic GLS pathway are suppressed.

Through a similar protocol, we have previously also analysed the full-transcriptomic responses of *Arabidopsis* and sweet pepper plants to WFT (Sarde *et al.* 2019; Steenbergen *et al.* 2019). A comparative transcriptomic analysis between the onion-thrips-induced white cabbage transcriptome and the WFT-induced *Arabidopsis* and sweet pepper transcriptomes showed 1) that the induction of JA biosynthesis and signalling is conserved in all three plant species, 2) that white cabbage responds rapidly with a relatively complex temporal transcriptional response (48 clusters), whereas the sweet pepper and *Arabidopsis* responses are somewhat slower and characterized by approximately half the number of clusters, i.e. 23 and 20 clusters respectively, 3) that TFs like MYB, bHLH and WRKY are conserved as major regulators of the response to thrips feeding, and 4) that the majority of the full-genome transcriptional responses against thrips are system-specific.

Majority of transcriptomic responses to thrips are system-specific

In both transcriptomic comparisons (white-cabbage vs *Arabidopsis* and white cabbage vs sweet pepper), a similar pattern in terms of the distribution of orthologous genes over the three categories (a) similarly regulated, (b) oppositely regulated and (c) differentially regulated in only one of the two species, was observed (Fig. 6 and 7). The emerging pattern is that: 1) there is more overlap in upregulated orthologous genes than in downregulated orthologous genes and 2) the majority of DEGs from each plant species shows species-specific differential expression. A similar conclusion was drawn for a comparison of *Arabidopsis* vs tomato plants upon infestation by the spider mite *Tetranychus urticae*, another cell-content feeder (Martel *et al.*, 2015) and the comparison of the *Arabidopsis* and sweet pepper transcriptomes in response to WFT feeding (Sarde *et al.*, 2019; Steenbergen *et al.*, 2019). Moreover, in the comparison between white cabbage (Brassicaceae) and *Arabidopsis* (Brassicaceae) a larger number of orthologues and common genes for each subset were found than in the comparison between white cabbage (Brassicaceae) and sweet pepper (Solanaceae). This suggests that the transcriptional responses are mostly plant-family specific, irrespective of different thrips species feeding. Moreover, the common subset

of upregulated genes from both comparisons represents GO terms associated with JA-related biological processes ("JA mediated signalling pathway" and "Response to JA"), validating conservation of JA-regulated defences in *Arabidopsis*, white-cabbage and sweet pepper, although elicited by two different thrips species. Overall, this analysis suggests a conservation of the JA pathway in all three plants, the presence of more commonalities in induced defences than suppressed processes and the majority of induced responses against thrips being system-specific.

Conserved activation of hormonal and secondary metabolite pathways upon thrips feeding

In plant-insect interactions, phytohormones are central players in plant responses to feeding damage (De Vos *et al*., 2005; Pieterse *et al*., 2009; Verhage *et al*., 2010; Pieterse *et al*., 2012; Stam *et al*., 2014). For example, JA is conserved in mediating responses against chewing caterpillars (Reymond *et al*., 2004; De Vos *et al*., 2005) and cell-content feeding insects like thrips (Abe *et al*., 2008; Abe *et al*., 2012; Sarde *et al*., 2018a; Steenbergen *et al*., 2018), whereas SA mediates responses against phloem-feeding insects like aphids and whiteflies (Zhu-Salzman *et al*., 2004; Walling, 2008; Pieterse *et al*., 2012; Tzin *et al*., 2015; Broekgaarden *et al*., 2018). In the present study, through comparative analysis, we show that both JA and ET hormonal pathways are induced in all three plant species (Table 1). This suggests conservation of synergism between the JA and ET hormonal pathways in fine tuning the responses to thrips. Moreover, several studies have shown induction of the SA pathway upon thrips feeding, but at later time points (Abe *et al*., 2008; Sarde *et al*., 2018a). In our studies, the SA pathway is also induced later in the brassicaceous plants *Arabidopsis* and white cabbage and supressed in sweet pepper during the first 8 h of thrips feeding. The relatively slow transcriptional response of sweet pepper may imply that the SA pathway is induced at later time points beyond the 8 h time window of the study (Sarde *et al.* 2019). The phenylpropanoid and flavonoid pathways are induced in all three plant species upon thrips feeding (Table 1), suggesting that these pathways may be involved in plant defence against thrips, as reported for defences against other insect herbivores (Mallikarjuna *et al*., 2004; Misra *et al*., 2010; Onkokesung *et al*., 2014).

Potential defensive role of indolic GLS in white cabbage against onion thrips

Although the role of glucosinolates in plant defence responses against herbivory is well studied, relatively little is known about their role against cell-content feeders, such as thrips. Glucosinolates, are a Brassicaceae-specific group of secondary metabolites. Upon herbivore feeding, the production of GLS is induced, resulting in interference with the performance of herbivorous insects. Different types of GLS are induced upon herbivory by different species, subsequently affecting their performance.

For example, indolic GLS affect the performance of phloem feeders (Elbaz *et al.*, 2012; Markovich *et al.*, 2013; Zust & Agrawal, 2016), whereas, aliphatic GLS affect the performance of chewing insects (Schweizer *et al.*, 2013). Our transcriptional data show that all genes involved in the regulation and biosynthesis of indolic GLS are induced. In contrast, the main upstream regulatory gene of aliphatic GLS biosynthesis, *BoMYB28*, is downregulated and none of the genes involved in biosynthesis of aliphatic GLS showed differential expression upon thrips feeding. A similar pattern of induction in indolic GLS and suppression of aliphatic GLS is seen in the WFT-induced *Arabidopsis* transcriptome (Fig. S2). This suggests specific induction of indolic GLS in brassicaceous plants by thrips feeding and indicates their potential role in defence against them. Similar observations of specific induction of indolic GLS and their defensive role are reported in *Arabidopsis* in response to another cell-content feeder, the two-spotted spider mite *Tetranychus urticae* (Zhurov *et al.*, 2014). These data suggest that the induction of indolic GLS upon damage by cell-content feeders and their potential defensive role is conserved in different brassicaceous species.

Rapid and complex transcriptional response of white cabbage to onion thrips feeding

Plants respond to different stresses by dynamically reprogramming their transcriptome (Windram *et al.*, 2012; Lewis *et al.*, 2015; Hickman *et al.*, 2017; Sarde *et al.*, 2019; Steenbergen *et al.*, 2019). In plant-insect interactions, the speed and complexity of the transcriptional response of plants depends on many characteristics of the attacking herbivore, such as feeding guild, density of herbivores and timing of attack (Heidel-Fischer *et al.*, 2014; Stam *et al.*, 2014). Until now, several studies on plant transcriptomic responses to different herbivore species analysed the response at only one or two time points spread over a period of 24 h or longer (De Vos *et al.*, 2005; Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011; Zhang *et al.*, 2013; Appel *et al.*, 2014; Diaz-Riquelme *et al.*, 2016; Kroes *et al.*, 2017; Broekgaarden *et al.*, 2018; Tu *et al.*, 2018). The main focus of these studies was to understand the overall response to feeding by the respective herbivore. In the present study, by comparing the high-density whole-genome transcriptional response of three plant species (*Arabidopsis*, sweet pepper and white cabbage) to thrips feeding, we show that white cabbage is relatively fast in its overall transcriptional response (Table 1). In white cabbage, the majority of the DEGs are induced within 1-2 h (Fig. 5 and 8), whereas in *Arabidopsis* and sweet pepper this is later than 2-3 h of thrips feeding (Fig. 8) (Sarde *et al.*, 2019; Steenbergen *et al.*, 2019). The rapid response of white cabbage comprises genes involved in the biosynthesis of phytohormones and secondary metabolites (Fig. 3, 4 and Table 1). Whether this is a plant-specific characteristic or a thrips-specific characteristic remains to be investigated. If it is a thrips-specific characteristic, the relatively rapid response of white cabbage against onion thrips compared to *Arabidopsis* and

sweet pepper against WFT feeding might be due to two main reasons: 1) onion thrips effectors are less effective in suppressing the initial defence response of white cabbage or, 2) onion thrips feed faster than WFT. Moreover, we found more temporally dynamic transcriptional patterns (genes in clusters with similar expression pattern) among the DEGs of white cabbage (48 clusters), compared to *Arabidopsis* (20 clusters) (Steenbergen *et al.*, 2019) and sweet pepper (23 clusters) (Sarde *et al.*, 2019). The larger number of temporally dynamic transcriptional patterns may be indicative of the complexity of the transcriptional response of white cabbage to onion thrips feeding. This relatively complex transcriptional response of white cabbage could be a result of its evolution from multiple ancestral polyploidy events or from an overall larger number of genes (Parkin *et al.*, 2014). Taken together, this comparative analysis suggests that white cabbage responds rapidly with a complex transcriptional pattern to onion thrips feeding.

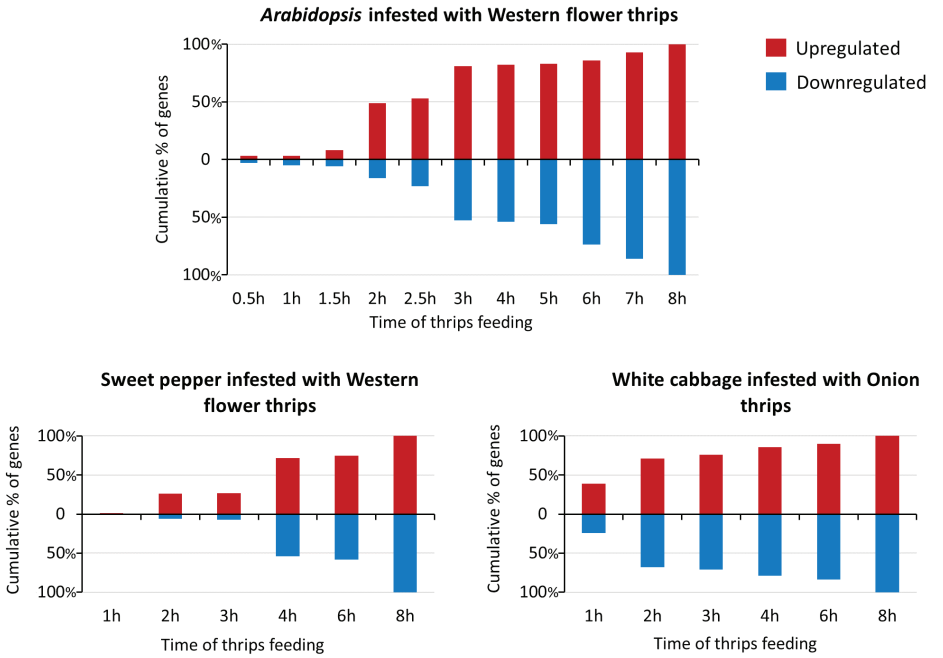

Figure 8. Whole-genome transcriptional response of *Arabidopsis*, white cabbage and sweet pepper in response to thrips feeding. 100% is 2788 (1820 up- and 968 downregulated) genes for *Arabidopsis*, 3062 (2060 up- and 1002 downregulated) genes for sweet pepper and 5799 (3790 up- and 2009 downregulated) genes for white cabbage.

Table 1. Comparison of temporal responses of phytohormonal and secondary metabolite pathways in WFT-induced *Arabidopsis* and sweet pepper and onion-thrips-induced white cabbage transcriptome. Red and blue colours represent up- and downregulation, respectively. BP, biological processes; TP, Time points.

	Sweet pepper						White cabbage						*Arabidopsis*									
TP / BP	1h	2h	3h	4h	6h	8h	1h	2h	3h	4h	6h	8h	0.5h	1h	1.5h	2h	2.5h	3h	4h	5h	6h	7h	8h
JA																							
ET																							
SA																							
Phenylpropanoids and flavonoids																							
Isoprenoids (Terpenoids)							--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Indole GLS	--	--	--	--	--	--																	
Aliphatic GLS	--	--	--	--	--	--																	
Indirect defence/GLVs																							

Conservation of bHLH, MYB and WRKY TFs as major regulators upon thrips feeding

TFs modulate the transcriptional reprogramming of plants in response to different stresses (Breeze *et al.*, 2011; Windram *et al.*, 2012; Jin *et al.*, 2017; Sarde *et al.*, 2019; Steenbergen *et al.*, 2019). In the white cabbage transcriptomic response to onion thrips and in the *Arabidopsis* and sweet pepper transcriptomic responses to WFT, we found TF families such as bHLH, MYB and WRKY being overrepresented in all three plant species. Moreover, MYB and bHLH TFs are also found to be overrepresented in the *Arabidopsis* transcriptomic response to exogenous MeJA application (Hickman *et al.*, 2017). This suggests that these TF families are conserved in all three plant species in regulating the defences against thrips species mediated by JA. Additionally, in all three plant species, through motif enrichment analysis, bHLH TFs are found to be enriched in gene clusters overrepresented in JA-related biological processes (Sarde *et al.*, 2019; Steenbergen *et al.*, 2019). This supports the role of the bHLH TF family in regulating JA signalling processes (Goossens *et al.*, 2017).

Conclusion

High-density transcriptomic analysis of the onset of plant-thrips interactions reveals the complexity of responses of three plant species to feeding by these herbivores. Almost a tenth of a plant's genome changes transcription level in with many groups of genes that share their dynamic expression pattern. The transcriptional response is highly specific for each plant-thrips interaction. More similarities were recorded among upregulated genes than among downregulated genes. The three major phytohormones, JA, SA and ET show similar expression patterns albeit at different temporal scales. Similarities among upregulated genes were especially related to JA biosynthesis and responses. This detailed comparative transcriptomic analysis of early molecular processes underlying plant-thrips interactions underlines the complex choreography of induced plant responses to insect feeding.

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Supplementary figures

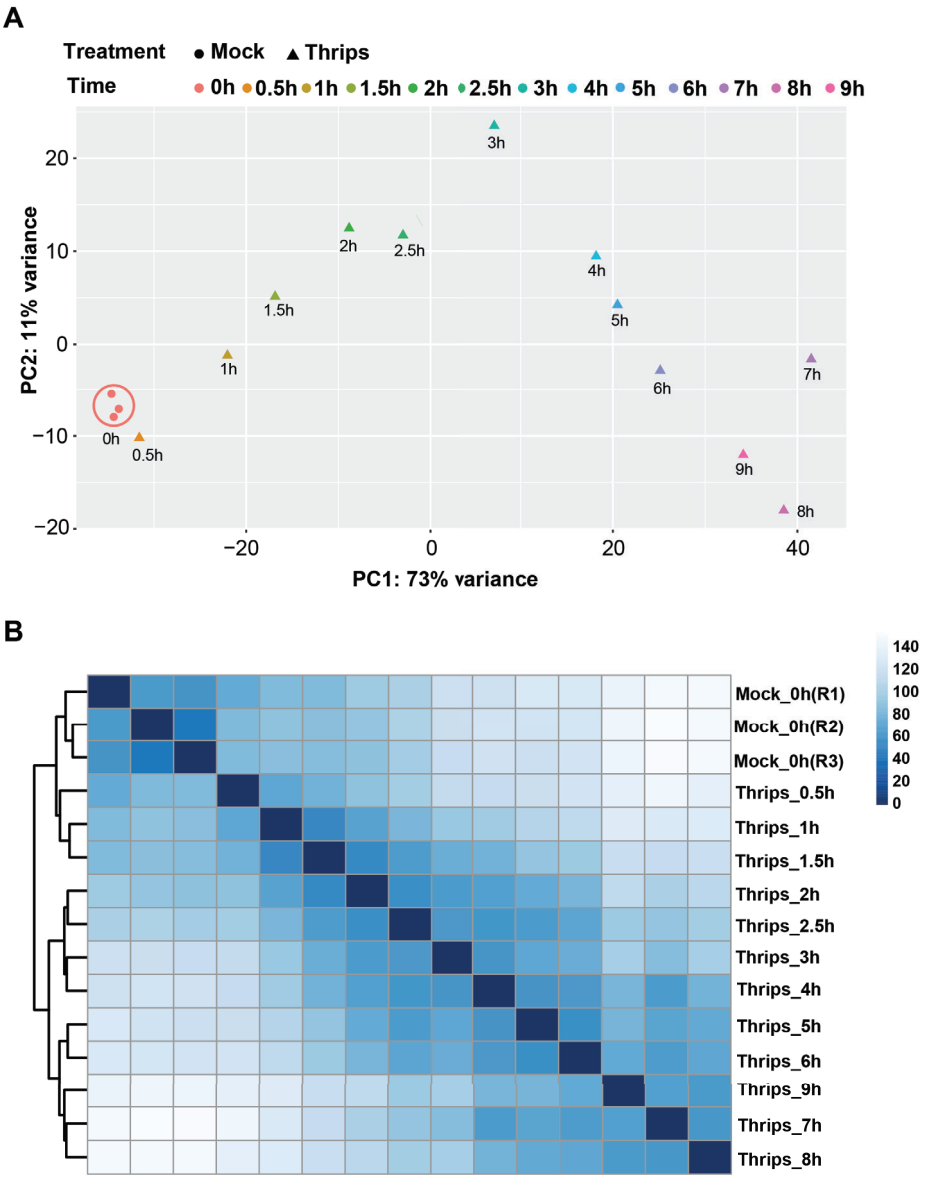

Figure S1. **PCA and sample-to-sample distance analysis of transcriptome of white-cabbage infested (thrips) and non-infested (control/mock) plants. (A)** PCA plot of sweet pepper non-infested (mock) and infested (thrips) transcriptome. PCA was generated on the regularized log_2-transformed data with DESeq2 package in R. Colours and shapes indicate different time points and treatments, respectively. Variation in percentage within the samples is depicted on both axes. **(B)** Sample-to-sample plot of sweet pepper non-infested (mock) and infested (thrips) transcriptome. It was generated on the regularized log_2-transformed data with DESeq2 package in R.

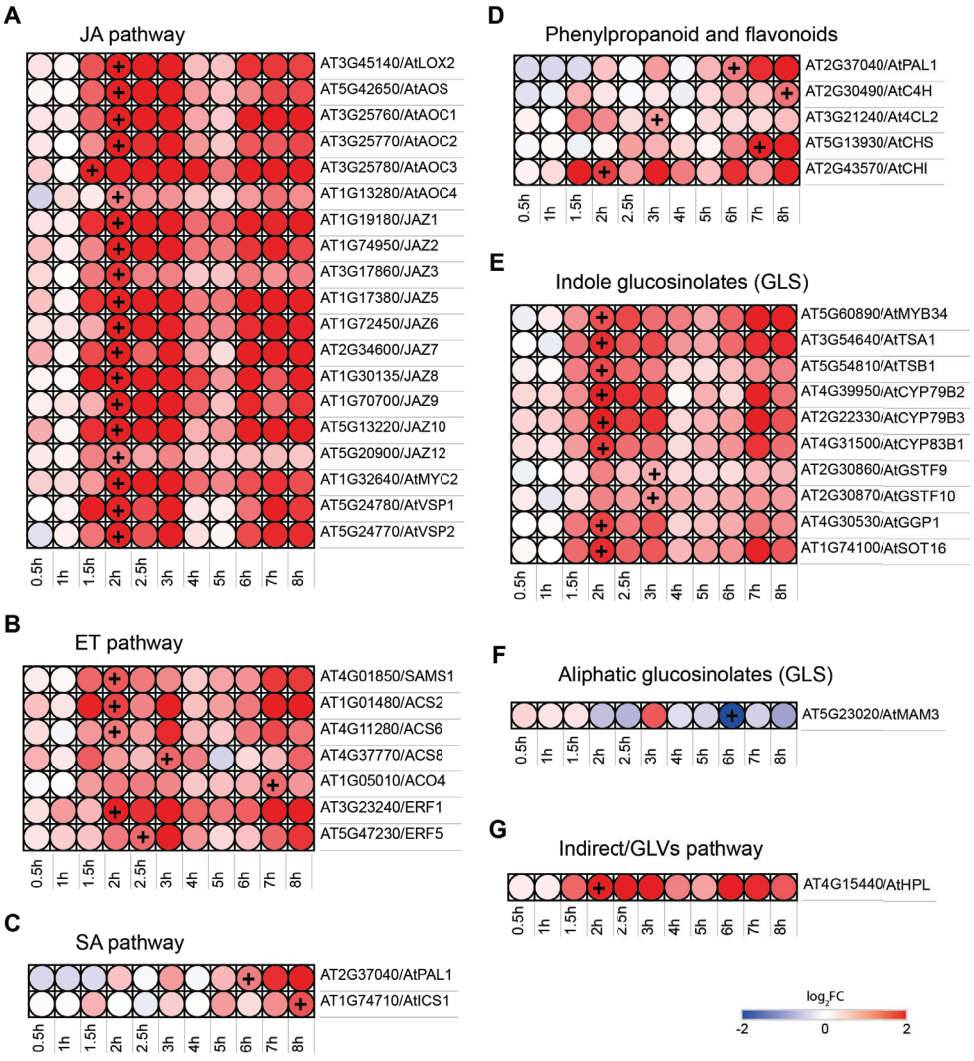

Figure S2. Expression pattern of *Arabidopsis* genes involved in biosynthesis of hormonal, secondary metabolites and indirect defences. (A) JA pathway, **(B)** ET pathway, **(C)** SA pathway, **(D)** phenylpropanoid and flavonoid pathways, **(E)** Indole glucosinolates (GLS), **(F)** Aliphatic glucosinolates (GLS), **(G)** Green-leaf volatiles (GLVs). '**+**' sign indicates significant ($P < 0.01$) first time of differential expression (ftode) of each gene. Based on data from Steenbergen *et al.* (2019).

Chapter 6

General discussion

Introduction

Due to their sessile nature, plants constantly face attack from mobile herbivorous insects. In comparison to other biotic stresses, insect herbivores are the most species-rich group of plant attackers (Schoonhoven *et al.*, 2005). To defend themselves, plants have evolved constitutive (always present) and induced defences (elicited by attack). Upon herbivore recognition, a plant induces defence mechanisms by activating signal transduction pathways that initiate the transcriptional responses. Subsequently, the transcriptional responses are dynamically reprogrammed resulting in the dynamic activation or deactivation of several biological processes (Windram *et al.*, 2012; Hickman *et al.*, 2017; Sarde *et al.*, 2019; Steenbergen *et al.*, 2019). This reorganization of transcriptional and biological processes causes extensive reprogramming of plant phenotype (Stam *et al.*, 2014), that impacts the ecology of plants during the season (Poelman *et al.*, 2010) or over seasons (Stam *et al.*, 2018). Thus, unravelling transcriptional responses of plants in response to insect herbivory can shed light on the mechanisms underlying the dynamics of plant phenotype expression. In this thesis I have focussed on cell-content feeding thrips as insect herbivores. To unravel the details of thrips-inducible defence mechanisms, in this project I focused on elucidating the underlying mechanisms and temporal transcriptional responses of plants to thrips feeding.

To capture transcriptional responses of plants to insect herbivory, microarray and RNA-Seq analyses are extensively used. Nowadays, due to the decrease in the price of sequencing, the RNA-Seq technique is gaining popularity over microarray analysis (Heidel-Fischer *et al.*, 2014). Moreover, RNA-Seq has several advantages over microarray analysis, such as 1) no background information on the genome is needed, 2) it offers high depth coverage in sequencing, 3) less complex normalization methods, 4) high accuracy and 5) detection of gene-splicing variants (Wang *et al.*, 2009; Ozsolak & Milos, 2011; Van Verk *et al.*, 2013). A growing body of studies have analysed transcriptomic responses of plants against different insect herbivores (De Vos *et al.*, 2005; Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011; Zhang *et al.*, 2013; Appel *et al.*, 2014; Coolen *et al.*, 2016; Davila Olivas *et al.*, 2016; Diaz-Riquelme *et al.*, 2016; Kroes *et al.*, 2017; Broekgaarden *et al.*, 2018; Tu *et al.*, 2018). These studies were performed for a limited number of time points since the start of herbivory, because they aimed mainly at elucidating the overall transcriptional responses and associated secondary metabolites, thus providing a low-resolution temporal representation of plant transcriptomic responses. To gain more insight into how plants temporally reprogram their transcriptome, performing high-density time-series experiments of transcriptional responses to insect herbivory is crucial.

This project was part of a research programme which aimed to elucidate underlying genetic mechanisms of responses of sweet pepper to Western flower thrips (WFT) feeding and dynamics of transcriptional responses of *Arabidopsis* and sweet pepper to WFT feeding and white cabbage to onion thrips feeding. Several approaches, such as genomics, phylogenetics, high-density transcriptomics and behavioural studies were used in this thesis. To gain a high-resolution portrait of sweet pepper and white cabbage transcriptomic responses to thrips feeding, a state-of-the-art next generation transcriptomics/bioinformatics approach was implemented.

Prominent role of LOX-mediated JA pathway in defence response against thrips

In response to insect herbivory, plants activate phytohormonal pathways that regulate a suite of responses, including the biosynthesis of secondary metabolites and defence-related proteins. Based on the feeding mode of insects, plants trigger different phytohormonal pathways, such as the jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) pathways, that regulate specific defences against different types of insect herbivory (Pieterse *et al.*, 2009; Verhage *et al.*, 2010; Pieterse *et al.*, 2012; Stam *et al.*, 2014). For example, responses to chewing insects like caterpillars (Reymond *et al.*, 2004; De Vos *et al.*, 2005) and cell-content feeding insects like thrips (Abe *et al.*, 2008; Abe *et al.*, 2009; Steenbergen *et al.*, 2018) are mediated by the JA pathway, while responses to phloem-feeding insects like aphids and whiteflies are mediated by the SA pathway (Zhu-Salzman *et al.*, 2004; Walling, 2008; Pieterse *et al.*, 2012; Tzin *et al.*, 2015; Broekgaarden *et al.*, 2018).

JA biosynthesis occurs via several enzymatic steps, among which oxygenation of linolenic acid is the critical step to initiate the biosynthesis of oxylipins, such as jasmonates and green leaf volatiles (GLVs) that are known to be involved in defence responses against insect feeding (Brash, 1999; Feussner & Wasternack, 2002; Allmann *et al.*, 2010; Yan *et al.*, 2013; Losvik *et al.*, 2017). The lipoxygenase (*LOX*) gene family mediates several biological processes, such as fruit ripening, tuber development, seed germination and plant defences (Brash, 1999; Feussner & Wasternack, 2002). By oxygenating linolenic acid (Kolomiets *et al.*, 2001; Bailly *et al.*, 2002; Feussner & Wasternack, 2002; Kessler, 2004; Barry & Giovannoni, 2007; Yan *et al.*, 2013), plants activate JA-regulated defences in response to insect feeding. Information on this gene family has been reported for several plant species, such as *Arabidopsis* (Umate, 2011), tomato, kiwifruit (Zhang *et al.*, 2006), olive (Padilla *et al.*, 2009, 2012), melon (Zhang *et al.*, 2014), cucumber (Liu *et al.*, 2011) and grapevine (Podolyan *et al.*, 2010). In chapter 2, the lipoxygenase (*LOX*) gene family from pepper (*Capsicum annuum*) has been identified and classified. In addition, based on in-depth *in-silico* and expression analysis, the functions of identified pepper *LOXs* were predicted.

From the reported multiple *LOXs* in different plant species, usually one 13-*LOX* is involved in induced resistance of plants to insect herbivory via biosynthesis of JA and thus activating the JA signalling pathway. Several plant species show reduced resistance to insects, when the expression of that specific 13-*LOX* was disrupted; for example, *AtLOX2* in *Arabidopsis* (Bell *et al*., 1995), *SlLOXD* (*TomLOXD*) in tomato (Yan *et al*., 2013), *NaLOX3* in tobacco (Halitschke & Baldwin, 2003; Kessler, 2004) and *StLOXH3* in potato (Royo *et al*., 1996). In chapter 3, through *in-silico* analysis, I narrowed down the *LOX* genes to a specific 13-*LOX* gene (*CaLOX2*) in pepper, and experimentally characterized its functional role in the JA-biosynthetic pathway, by silencing it through the Virus-Induced Gene Silencing (VIGs) technique. I subsequently showed that knocking down *CaLOX2* made the plant more susceptible to WFT. Data presented in chapters 4 and 5, on the induction of the whole JA cascade including 13-*LOXs* in sweet pepper and white cabbage upon WFT and onion thrips feeding, respectively, consolidates the prominent role of lipoxygenase-mediated JA pathway in the response of different plant species to thrips feeding.

Extensive transcriptional reprogramming of host plants upon thrips feeding

One of the global quantifiable measures of plant responses to insect herbivory is to capture the set of differentially expressed genes (Heidel-Fischer *et al*., 2014). Plants extensively reprogram their transcriptome with time in response to several biotic and abiotic stresses (Breeze *et al*., 2011; Windram *et al*., 2012; Hickman *et al*., 2017). So far, several studies have captured transcriptomic responses of plants to insect herbivory limited to one or two time points (De Vos *et al*., 2005; Ehlting *et al*., 2008; Bidart-Bouzat & Kliebenstein, 2011; Zhang *et al*., 2013; Appel *et al*., 2014; Diaz-Riquelme *et al*., 2016; Kroes *et al*., 2017; Broekgaarden *et al*., 2018; Tu *et al*., 2018), with a focus on the identification of the differentially expressed genes and their involvement in the biosynthesis of different metabolites, but not the dynamics of the transcriptional reprogramming. In the studies presented in chapters 4 and 5, I unravelled the temporally differential expression of large numbers of genes in sweet pepper and white cabbage plants upon WFT and onion thrips feeding, respectively. A similar pattern of transcriptional reprogramming was observed in *Arabidopsis* in response to WFT feeding (Steenbergen *et al*., 2019). The diversity of temporal expression patterns of numerous genes in all three plants is an indication of the extensive transcriptional reprogramming that occurs in plants upon thrips feeding.

Speed, intensity and complexity of transcriptional response is plant- and stress-specific

Transcriptional reprogramming forms the basis of plant response to insect herbivory (Heidel-Fischer *et al*., 2014). Apart from overall identifying differentially expressed genes in a plant's transcriptomic response, the dynamics of transcriptional respons-

es can also be assessed in several other ways, such as the speed, intensity and complexity of the response. The speed of transcriptional response can be measured based on the number of genes showing differential expression at different time points since the onset of the stress, whereas intensity and complexity can be determined by the overall number of differentially expressed genes (DEGs) and the number of expression patterns observed among all DEGs, respectively. Although several studies have performed transcriptomic studies in response to insect herbivory, not much is revealed about temporal transcriptional reprogramming as the studies were performed for only a few time points (De Vos *et al.*, 2005; Ehlting *et al.*, 2008; Bidart-Bouzat & Kliebenstein, 2011; Zhang *et al.*, 2013; Appel *et al.*, 2014; Diaz-Riquelme *et al.*, 2016; Kroes *et al.*, 2017; Broekgaarden *et al.*, 2018; Tu *et al.*, 2018). To our knowledge, few studies have performed high-density time-series experiments to capture the temporal transcriptional reprogramming of plants against biotic and abiotic stresses. For example, leaf senescence in *Arabidopsis* (22 time points over a period of 39 days) (Breeze *et al.*, 2011), response of *Arabidopsis* to *Botrytis cinerea* infection (24 time points over a period of 48 hours) (Windram *et al.*, 2012) and *Arabidopsis* response upon exogenous JA application (15 time points over a period of 16 hours) (Hickman *et al.*, 2017) have been studied.

In chapters 4 and 5, I investigated the differences in transcriptional responses between WFT-infested *Arabidopsis* (Steenbergen *et al.*, 2019) and sweet pepper as well as onion-thrips-infested white cabbage plants. In these studies, I observed that the transcriptional response of white cabbage plants is characterized by a higher speed, intensity and complexity compared to the response of *Arabidopsis* and sweet pepper plants to thrips feeding. The transcriptional response of *Arabidopsis* to *B. cinerea* infection showed a higher magnitude (9838 DEGs representing ca 30 % of the CATMA v3 microarray) and complexity (44 gene clusters) in transcriptional response, but a slow speed of response (Windram *et al.*, 2012). Similar observations (6323 DEGs representing ca 22 % of the CATMA v3 microarray and 48 clusters) were reported during leaf senescence of *Arabidopsis* (Breeze *et al.*, 2011). Moreover, upon exogenous JA application to *Arabidopsis,* the transcriptional response was relatively rapid, but less intense (3611 DEGs representing ca 10 % of the *Arabidopsis* genome (TAIR version 10)) and complex (27 clusters) (Hickman *et al.*, 2017) than for *Arabidopsis* in response to thrips feeding. The high magnitude and complexity of the transcriptional response of *Arabidopsis* to leaf senescence and *B. cinerea* infection could be due to the slow speed of these stresses. In contrast, exogenous JA application and thrips feeding, both relatively fast inducers, show relatively rapid and less intense transcriptional responses in plants. Nevertheless, the response of white cabbage plants against onion thrips was relatively rapid, intense and complex compared to the responses of *Arabidopsis* and sweet pepper to WFT. Taken together,

comparative investigations of the speed, intensity and complexity of transcriptional responses will be interesting to gain understanding of the repertoire of plant responses to environmental stresses.

Majority of transcription factors activated by JA are plant and stress-specific

In response to different biotic and abiotic stresses, plants activate signal-transduction pathways that regulate defence responses. This includes induction of transcription factors (TFs), secondary metabolites and defence-related proteins. Phytohormones such as JA, SA and ET are central players in modulating defence responses against insect herbivory and pathogens (De Vos *et al*., 2005; Verhage *et al*., 2010; Erb *et al*., 2012; Pieterse *et al*., 2012; Stam *et al*., 2014), whereas abscisic acid is important in responses to abiotic stresses such as heat, drought and cold (Yamaguchi-Shinozaki & Shinozaki, 2006; Kilian *et al*., 2007; Huang *et al*., 2008).

In chapters 4 and 5, I document conservation and specificities of TF families in *Arabidopsis* (Steenbergen *et al*., 2019) and sweet pepper (chapter 4) induced upon WFT feeding and white cabbage (chapter 5) induced upon onion thrips feeding. We found TF families such as bHLH, MYB and WRKY being overrepresented in all three plant species, with several others TF families specifically induced in each plant species. This suggests that, although JA is a prominent phytohormone regulating defence responses against thrips feeding, the majority of TF families activated by JA are different in *Arabidopsis*, sweet pepper (chapter 4) and white cabbage plants, regulating different defence mechanisms. Furthermore, in *Arabidopsis,* few studies showed significant induction of certain TF families in response to leaf senescence (Breeze *et al*., 2011), *B. cinerea* infection (Windram *et al*., 2012) and exogenous JA application (Hickman *et al*., 2017). Leaf senescence is known to activate several stress-related phytohormones, such as JA, SA and ABA (Weaver *et al*., 1998; Morris *et al*., 2000; He *et al*., 2002) whereas, *B. cinerea* activates the JA/ET signalling pathway (Thomma *et al*., 1998; Rowe *et al*., 2010). Surprisingly, more common TF families were induced in leaf senescence and *B. cinerea* stresses (WRKY, NAC, AP2-EREBP, and C3H) compared to exogenous JA application (bHLH, ERF, and MYB). In contrast, TF families induced upon exogenous application of JA are also induced in *Arabidopsis* (bHLH and MYB) (Steenbergen *et al*., 2019), sweet pepper (bHLH, ERF, and MYB) and white cabbage plants (bHLH, ERF, and MYB). Taken together, although JA is a central player in regulating transcriptional responses against *B. cinerea*, thrips feeding and leaf senescence, apart from a few TF families, the majority of TF families are induced in a plant or stress specific manner suggesting JA activates different TF families in different plant species to regulate specific defence responses.

Selection of time points for assessing transcriptional response of plants to herbivore feeding

In the past decade, considerable progress has been made to quantify plant transcriptional responses to feeding by herbivorous insects. In plants, the field of transcriptomics has made much progress with advanced technologies such as whole-genome microarray or RNA-Seq, since the early use of a microarray which elucidated differential transcription of 45 transcripts in *Arabidopsis* (Schena *et al.*, 1995). Nowadays, due to low costs of next generation sequencing technologies (RNA-Seq), transcriptional profiling of non-model plant species can be carried out for responses to any insect species. Several studies have captured transcriptional responses of plants against different insect herbivory, but the selection of time points seems to have usually been done without extensive knowledge of temporal patterns in transcriptional responses. This random selection of time points may lead to missing a significant number of transcripts or missing the temporal dynamics of the transcriptional response, which may fluctuate especially at the onset of the plant's response. Therefore, to enhance the insight into the transcriptional responses of plants, it is not only important to make a high-density temporal assessment, but also to select the most relevant time points. Selection of the most relevant time points can help to capture the maximum transcriptional response in terms of number of differentially expressed genes as well as the dynamics of the complex response. In chapters 3 and 4, due to monetary constraints we employed a strategy to select the most representative time points from a total of 13 time points within a period of the initial 9 hours of thrips herbivory. Based on initial sequencing of one replicate from 12 time points (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 hours of infestation) and subsequent *in-silico* analysis, six time points (excluding the 0 h time point) were selected and finalised to capture the transcriptomes of sweet pepper and white cabbage. Therefore, to capture maximum transcriptional responses of plants against insect herbivory, implementation of selection of time points in an experimental setup is of utmost important.

Future perspectives

Thrips are serious pests on various ornamental and food crops (Steenbergen *et al.*, 2018), significantly reducing yield. There is considerable literature on induced defences in response to chewing and phloem-feeding insects compared to cell-content feeders. Likewise, several *R*-genes were successfully identified in different plants mostly against phloem feeders (Broekgaarden *et al.*, 2011). High-resolution transcriptional data presented in this thesis can be explored to build gene-regulatory networks (GRNs), to select potential regulators (TFs) of or candidate genes providing resistance against thrips feeding, that can be further tested for functional analysis

through overexpression or silencing. Those genes that are involved in the expression of the relevant phenotype can be used by breeders to develop thrips-resistant varieties. In addition to feeding damage, thrips also inflict indirect damage to plants by transmitting tospoviruses (Steenbergen *et al.*, 2018). Thrips feeding elicits especially JA-regulated defences (Li *et al.*, 2002; Abe *et al.*, 2008; Abe *et al.*, 2009; Sarde *et al.*, 2018) (chapter 4 and 5), whereas, contrastingly, TSWV elicits SA-regulated defences (Abe *et al.*, 2012). The antagonistic relation between the JA and SA pathways is well-known (Pieterse *et al.*, 2012). Therefore, it would be interesting to compare the thrips-induced transcriptional response to the transcriptional response induced by viruliferous thrips. This can lead to understanding of the dynamics of transcriptomic response under more relevant conditions that can be a first step towards thrips and tospovirus-resistant crop varieties.

Furthermore, in the natural environment, plants encounter different biotic and abiotic stresses that occur in sequence or simultaneously. Few studies have investigated plant responses to combination of insect herbivory are different from plant responses to single insect herbivory (Pieterse & Dicke, 2007; Dicke *et al.*, 2009; Utsumi *et al.*, 2010). Recent studies reported differences in phenotypic and transcriptomic plant responses to combined and single biotic and abiotic stresses (Coolen *et al.*, 2016; Davila Olivas *et al.*, 2016). Therefore, to understand and develop crop varieties possessing resistance to several stresses, studying transcriptional responses to combinations of stresses is important. Moreover, recent evidence shows how plant ontogeny influences the resistance level of plants, and especially the transition from vegetative stage to the flowering stage may alter plant resistance responses (Boege & Marquis, 2005; Barton & Koricheva, 2010; Diezel *et al.*, 2011). It will be interesting to compare the whole-genome transcriptional responses of plants to thrips feeding in the vegetative and flowering stages. This may yield insights into the switch that occurs in plant defence mechanisms from vegetative to flowering stage including core TFs, and specialized metabolites.

Conclusion

In this thesis, I have elucidated underlying genetic mechanisms of sweet pepper in response to WFT feeding. By identifying and functionally characterizing the role of *CaLOX2*, I consolidated the importance of JA-regulated defences against thrips in sweet pepper. Furthermore, through high-density time-series RNA-Seq analysis of leaf tissue at 12 time points within the first 9 hours, I captured the dynamics of the early transcriptional response of sweet pepper and white cabbage plants to WFT and onion thrips feeding, respectively. Overall, the data represent a conservation of induction of JA, ET and phenylpropanoid and flavonoid pathways in sweet pepper and

white cabbage. Furthermore, the genes involved in the biosynthesis of indolic glucosinolates were induced in white cabbage, whereas genes involved in biosynthesis of isoprenoids (terpenoids) were induced in sweet pepper. Comparative transcriptomics of the WFT-induced response of *Arabidopsis* and sweet pepper and the onion-thrips induced response of white cabbage plants suggests that the majority of transcriptomic responses against thrips are system-specific. Moreover, in comparison to *Arabidopsis* and sweet pepper, the transcriptomic response of white cabbage is more rapid and complex. This thesis provides a first impression of the complexity of early molecular aspects of thrips-plant interactions. The information generated will help to understand how plants defend themselves against these cell-content feeding insect herbivores.

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Summary

In nature, plants continuously face the attack from insect herbivores. To defend themselves, plants have evolved a plethora of direct and indirect defence mechanisms that are present either constitutively or induced upon insect herbivory. In response to insect herbivory, plants activate phytohormonal signal-transduction pathways, transcriptional responses and biological processes. These changes in transcriptional and biological processes level influence the plant's phenotype and consequently plant ecology during the current season or over seasons. To gain more insight into the dynamics of plant phenotype, elucidating transcriptomic responses to insect herbivory is critical. Nowadays, to capture transcriptional responses against insect herbivory, RNA-Seq technology is used extensively. Various studies have investigated whole-genome transcriptional responses of plants against different insect herbivores, but only few have reported on responses to cell-content feeding thrips. Thrips (Thysanoptera) are minute cell-content feeding insects and are serious pests on many commercial and ornamental plants. Thrips species such as western flower thrips (WFT) and onion thrips are among the most devastating pests on *e.g.* sweet pepper and white cabbage plants, respectively. Therefore, using a high-density time-series approach, the focus of this thesis was to investigate whole-genome transcriptional responses of sweet pepper and white cabbage plants upon WFT and onion thrips feeding, respectively, and the underlying genetic mechanisms. In addition, I focussed on one particular gene family, the lipoxygenases and a gene in this family that is involved in thrips-induced crop resistance.

Chapter 2 focused on the identification of the lipoxygenase gene family in pepper (*Capsicum annuum*). Lipoxygenases (LOXs) are non-heme, iron-containing dioxygenases involved in several developmental and defence-related plant processes, such as seed germination, fruit ripening, tuber development and JA-regulated plant defences. To identify this multi-gene family, several approaches were implemented, such as comparative genomics, sequence analysis, domain-scan analysis, phylogenetic analysis, homology modelling and transcriptional analysis. This approach resulted in the identification of a total of eight *LOX* genes in pepper classifying four LOXs (*CaLOX1*, *CaLOX3*, *CaLOX4* and *CaLOX5*) as 9-LOXs and four (*CaLOX2*, *CaLOX6*, *CaLOX7* and *CaLOX8*) as 13-LOXs. Furthermore, these approaches also showed high conservation of pivotal amino acids and dynamic expression profiles of 13-LOXs compared to 9-LOXs upon exogenous JA application and WFT feeding. From the results, the putative functions of two 13-LOXs, *CaLOX6* and *CaLOX7*, in the biosynthesis of Green Leaf Volatiles (GLVs) were predicted.

Chapter 3 further narrowed down the *LOX* gene-family to one lipoxygenase (*CaLOX2*) gene through *in-silico* analysis and functionally characterized its involvement in jasmonate-dependent induced defence against WFT. Here, I implemented a multidisci-

plinary approach which includes *in-silico*, transcriptional, behavioural and chemical analyses. The expression of JA-related marker genes (*CaLOX2* and *CaPIN II*) was induced upon thrips feeding. Silencing of *CaLOX2* in sweet pepper plants through virus-induced gene silencing (VIGS) significantly hampered the biosynthesis of the phytohormone JA and its derivatives. Subsequently, this resulted into reduced resistance and increased preference of thrips for these sweet pepper plants. Furthermore, thrips feeding ability, preference and population development were hampered upon exogenous JA application. Overall, this chapter shows that *CaLOX2* is involved in JA-mediated plant resistance to thrips feeding.

In response to insect feeding, temporal transcriptional reprogramming forms the basis of dynamically changing plant phenotype. Therefore, in **Chapter 4**, I focused on elucidating the dynamics of transcriptional reprogramming of sweet pepper leaf tissue in response to WFT feeding. For this, leaf tissue of sweet pepper plants that were subjected to WFT infestation was harvested at 12 time points within the first 9 hours of WFT feeding for RNA-Seq analysis. Approximately 8.6% (2060 up- and 1002 down-regulated) of the pepper genes were differentially expressed upon WFT feeding. Subsequent in-depth analysis of a selected set of 6 time points categorized the 3062 DEGs into 23 gene clusters (16 upregulated and 7 downregulated), each possessing a unique temporal expression pattern. Clusters of upregulated genes were associated with defence-related biological processes, whereas clusters of downregulated genes were associated with developmental processes. The transcription factor families ERF, MYB, NAC, bHLH and WRKY emerged as pivotal regulators in response to WFT feeding. The data show a chronological order in the activation of hormonal (JA, ET) and secondary metabolite (phenylpropanoids, flavonoids and terpenoids) pathways. Eventually, the comparative analysis of the WFT-induced transcriptional responses of *Arabidopsis* and sweet pepper plants to WFT feeding shows a conservation in the induction of the JA-pathway in both plants, whereas the majority of transcriptional responses are plant-specific. In addition, it also showed that relatively more similarities exist in upregulated genes compared to downregulated genes.

Chapter 5 focused on 1) elucidating whole-genome transcriptional reprogramming of white cabbage plants in response to onion thrips feeding and 2) comparative transcriptomics to disentangle similarities and differences in transcriptional responses between WFT-induced *Arabidopsis* and sweet pepper as well as onion-thrips-induced white cabbage. The data for all three plant species were collected through a similar approach. Approximately 9.7 % of the white cabbage genes show differential expression within 8 h of onion thrips feeding. Forty-eight (32 upregulated and 16 downregulated) gene clusters with similar expression patterns were detected among the DEGs, with upregulated clusters associated with defence and downregulated clus-

ters with development-related biological processes. Phytohormone-related processes (JA, ET and SA) and secondary metabolite (phenylpropanoids, flavonoids, green-leaf volatiles and indolic glucosinolates) biosynthesis genes were induced, whereas aliphatic glucosinolate biosynthetic genes were suppressed. Comparative analysis of the transcriptional responses of *Arabidopsis* and sweet pepper to WFT and of white cabbage to onion thrips showed 1) conservation of the JA biosynthesis and signalling pathways, 2) conservation of involvement of TF families, such as MYB, bHLH and WRKY in regulating responses, 3) that the majority of the transcriptional responses to thrips are system-specific, 4) that genes involved in indole glucosinolate biosynthesis are upregulated, whereas genes involved in aliphatic glucosinolate biosynthesis are downregulated in both brassicaceous plants *Arabidopsis* and white cabbage, 5) that the white-cabbage transcriptomic response to onion thrips is relatively rapid and complex compared to the WFT-induced *Arabidopsis* and sweet pepper transcriptomic responses.

Finally, in **Chapter 6**, I discuss the dynamics of plant transcriptional responses to several stresses. This includes the importance of the *LOX*-mediated JA pathway against thrips feeding and several aspects of plants transcriptional reprogramming to thrips and other stresses. The advantages of the selection of time points in a high-density time series approach, as implemented in this project are discussed. Furthermore, I make recommendations to enhance our knowledge of induced defences in plants. In conclusion, this thesis highlights the intensity and complexity of plant transcriptional responses to thrips feeding through a comparative approach, while focussing on the details of one important gene involved in induced defence against thrips.

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Curriculum Vitae

Sandeep J. Sarde was born on 27 September, 1986 in Mangrul, Maharashtra, India. Growing up with surroundings and family of farmers, his passion for agriculture has been extensively nurtured since childhood. After obtaining the Bachelor degree in Biotechnology with distinction, he pursued his Master's degree in Agrigenomics at the University of Kiel (CAU), Germany, with a focus on plant breeding, phytopathology and genomics in applied agriculture. He performed his Master's thesis entitled "Unravelling of the spliceosomal proteins encoded by fungal genomes" by implementing the approach of comparative genomics under the supervision of Prof. Dr. rer. nat. Frank Kempken and Dr. Abhishek Kumar. During the Master's programme, he was also involved in volunteer projects with Dr. Abhishek Kumar to determine the evolutionary history and genetic variants of different serpins in the vertebrates, particularly involved in blood-related diseases.

In January 2015, he started his PhD project in the Laboratory of Entomology at Wageningen University, The Netherlands. The PhD program was part of the TTW (former STW) consortium entitled "**G**reen defence **A**gainst **P**ests (GAP)". This GAP STW perspective program aimed to elucidate plant defence responses against cell-content feeding insects. The project was executed using multi-disciplinary approaches by the team effort of different Dutch universities and private agricultural companies such as, Rijk Zwaan, Enza Zaden, Syngenta, Keygene, East-West Seed and Bejo. In this consortium, Sandeep contributed to understanding of the dynamics of transcriptional responses of plants (sweet pepper, white cabbage and *Arabidopsis thaliana*) to thrips feeding and has presented his findings in this thesis.

Publication list

Published articles in a journal

Sarde SJ, Kumar A, Remme RN, Dicke M. **2018.** Genome-wide identification, classification and expression of lipoxygenase gene family in pepper. *Plant Molecular Biology* **98** (4-5): 375-387. **(Chapter 2 in this thesis)**

Sarde SJ, Bouwmeester K, Venegas-Molina J, David A, Boland W, Dicke M. **2018.** Involvement of sweet pepper *CaLOX2* in jasmonate-dependent induced defence against Western flower thrips. *Journal of Integrative Plant Biology*: 10.1111/jipb.12742. **(Chapter 3 in this thesis)**

Sarde SJ, Kumar A, Kempken F. **2018.** Spliceosomal proteins encoded by fungal genomes. *Current Science* **114**(8): 1677-1686.

Vaello T, **Sarde SJ**, Marcos-Garcia MA, de Boer JG, Pineda A**. 2018.** Modulation of plant-mediated interactions between herbivores of different feeding guilds: Effects of parasitism and belowground interactions. *Scientific Reports* **8**: 14424.

Steenbergen M, Abd-el-Haliem A, Bleeker P, Dicke M, Escobar-Bravo R, Cheng G, Haring MA, Kant MR, Kappers I, Klinkhamer PGL, Leiss KA, Legarrea S, Macel M, Mouden S, Pieterse CMJ, **Sarde SJ**, Schuurink RC, De Vos M, Van Wees SCM, Broekgaarden C. **2018.** Thrips advisor: exploiting thrips-induced defences to combat pests on crops. *Journal of Experimental Botany* **69**(8): 1837-1848.

Kumar A, Bhandari A, **Sarde SJ**, Goswami C**. 2017.** Ancestry & molecular evolutionary analyses of heat shock protein 47 kDa (HSP47/SERPINH1). *Scientific Reports* **7**: 10394.

Kumar A, Bhandari A, **Sarde SJ**, Muppavarapu S, Tandon R**. 2016.** Data on the evolutionary history of the V(D)J recombination-activating protein 1 - *RAG1* coupled with sequence and variant analyses. *Data in Brief* **8**: 87-92.

Kumar A, Bhandari A, **Sarde SJ**, Muppavarapu S, Tandon R. **2015.** Understanding V(D)J recombination initiator *RAG1* gene using molecular phylogenetic and genetic variant analyses and upgrading missense and non-coding variants of clinical importance. *Biochemical and Biophysical Research Communications* **462**(4): 301-313.

Kumar A, **Sarde SJ**, Bhandari A. **2014.** Revising angiotensinogen from phylogenetic and genetic variants perspectives. *Biochemical and Biophysical Research Communications* **446**(2): 504-518.

Kumar A, Bhandari A, **Sarde SJ**, Goswami C. **2014.** Genetic variants and evolutionary analyses of heparin cofactor II. *Immunobiology* **219**(9): 713-728.

Kumar A, Bhandari A, **Sarde SJ**, Goswami C. **2014.** Molecular phylogeny of C1 inhibitor depicts two immunoglobulin-like domains fusion in fishes and ray-finned fishes specific intron insertion after separation from zebrafish. *Biochemical and Biophysical Research Communications* **450**(1): 219-226.

Kumar A, Bhandari A, **Sarde SJ**, Goswami C. **2013.** Sequence, phylogenetic and variant analyses of antithrombin III. *Biochemical and Biophysical Research Communications* **440**(4): 714-724.

Articles in preparation

Sarde SJ, Hickman R, Steenbergen M, Wiegers G, Pieterse CMJ, Van Wees SCM, Dicke M. **2019.** Whole-genome transcriptional reprogramming of sweet pepper in response to Western flower thrips feeding. *Submitted*. **(Chapter 4 in this thesis)**

Sarde SJ, Hickman R, Steenbergen M, Wiegers G, Pieterse CMJ, Van Wees SCM, Dicke M**.** Comparative high-density transcriptomics reveals rapid and complex rearrangement of white cabbage transcriptome in response to thrips feeding. *In prep*. **(Chapter 5 in this thesis)**

Steenbergen M, Hickman R, **Sarde SJ**, Dicke M, Pieterse CMJ, Van Wees SCM**.** High-resolution temporal transcriptomic dynamics of *Arabidopsis thaliana* in response to Western flower thrips feeding. *In prep*.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Sandeep Jayaji Sarde
Date: 20 June 2019
Group: Laboratory of Entomology
University: Wageningen University & Research

1) Start-Up Phase	*date*
► **First presentation of your project**	
Plants-thrips Interaction: uncovering dynamics of the plant defense signaling network to identify novel leads for maximizing crop protection	21 April , 2015
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Subtotal Start-up Phase	**1.5**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student days, Soest	29 - 30 January, 2015
EPS PhD student days, Soest	28 - 29 January, 2016
EPS PhD student days, Soest	9-10 February, 2017
► **EPS theme symposia**	
EPS Theme 2 Symposium: Interactions between Plants and biotic agents, Utrecht	20 February, 2015
EPS Theme 2 Symposium: Interactions between Plants and Biotic Agents, Leiden	22 January, 2016
EPS Theme 1 Symposium: Developmental Biology of Plants, Leiden	28 February, 2017
EPS Theme 3 Symposium: Metabolism and Adaptation, Wageningen	14 March, 2017
► **Lunteren Days and other national platforms**	
ALW meeting Experimental Plant Sciences, Lunteren	13 -14 April, 2015
ALW meeting Experimental Plant Sciences, Lunteren	11-12 April , 2016
ALW meeting Experimental Plant Sciences, Lunteren	10-11 April , 2017
Annual meeting of Netherlands Entomological Society, Ede	15 December, 2017
Annual meeting of Netherlands Entomological Society, Ede	14 December, 2018
Green defence Against Pests (GAP) consortium meeting	21 April , 2015
Green defence Against Pests (GAP) consortium meeting	25 September, 2015
Green defence Against Pests (GAP) consortium meeting	6 April, 2016
Green defence Against Pests (GAP) consortium meeting	11 October, 2016
Green defence Against Pests (GAP) consortium meeting	18 May, 2017
Green defence Against Pests (GAP) consortium meeting	27 November, 2017
Green defence Against Pests (GAP) consortium meeting	13 March, 2018
Green defence Against Pests (GAP) consortium meeting	21 September, 2018
Green defence Against Pests (GAP) consortium meeting	29 November, 2018
► **Seminars (series), workshops and symposia**	
EPS seminar by Florian Schiestl: Evolution of floral signals in plants: mechanisms and consequences	12 March, 2015
EPS seminar by Alain Goossens: How jasmonates provide the key to harness plant chemistry	8 December, 2015
EPS seminar by Mark Varrelmann: The rhizomania complex in sugar beet – virus variation and resistance breaking	18 April, 2017
EPS seminar by Martin Cann: The immune receptor Rx1 remodels chromatin and chromatin interactors in immunity	11 July, 2017
EPS seminar by Sanjay Kapoor: Regulators of Reproductive Development in Rice	29 August, 2017

EPS seminar by U. Wyss: Highlights of hidden insect-worlds	2 October, 2017
EPS seminar by Asaf Levy: Bacteria and the future of agriculture: from sequence to function	22 February, 2018
WEES lecture by Kevin Foster: The evolution of cooperation and competition in microbes	22 January, 2015
WEES lecture by Hanna Kokko: Males exist. Does it matter	19 March, 2015
WEES lecture by Mart Krupovic: Natural history of viral capsids	23 February, 2017
WEES seminar by Alexie Papanicolaou: Identification of a new sensory neuron membrane gene and why phylogenomics is important	22 June, 2017
WEES seminar by Richard Lenski: Dynamics of Adaptation and Genome Evolution in a Long-Term Experiment	31 August, 2017
WEES seminar by Frantisek Marec: From sex chromosomes to sex determination in Lepidoptera	25 October, 2017
Ento-seminar: D.G. Stavenga, Colorful Insect Vision	22 February, 2016
Ento-seminar: Joop van Loon, Insect Nutrition	27 June, 2016
Ento-seminar: Eric Schranz, Genetics and Phylogeny	29 August, 2016
Ento-seminar: Sander Koenraadt, Ecology of vector-borne diseases	26 June, 2017
Ento-seminar: Chantal Vogels and Helen Esser, Arthropod-borne viruses	30 October, 2017
Ento-seminar: Katja Poveda, Intra and inter-specific trait variation in arthropods	15 February, 2018
Seminar by Douglas Mitchell: Genomics-enabled natural products discovery	31 March, 2016
Seminar by Eric Roalson : Cleomaceae: revising generic concepts and interpreting patterns of adaptation and diversification of C4 photosynthesis	4 May, 2016
Seminar by Nicole van Dam: Herbivore-induced plant volatiles accurately reflect evolutionary history, diet breadth, and feeding mode of herbivores	17 October, 2016
Seminar by Jennifer Doudna and Edze Westra: Rewriting our genes? CRISPR-CAS systems as tools for genome editing	30 September, 2016
Keygene seminar: Genotyping in the Seed & AgroFood Industry: today and in the future	26 January, 2017
Science Café Wageningen : Agriculture in a changing world	22 February, 2017
Promega seminar: Optimize and Analyze your RT-qPCR and RNA workflow	26 September, 2017
KeyGene and GENALICE seminar: Generate, Process, and Apply. Solutions for Agrigenomic big data challenges	30 November, 2017
11th Plant-Insect Interaction Workshop, Leiden	22 November, 2016
12th Plant-Insect Interaction Workshop, Wageningen	7 November, 2017
Wageningen PhD symposium: Diversity in Science, Wageningen	26 April, 2016
EPS Symposium: Goodbye of Ton Bisseling, Wageningen	8 February, 2017
Mini-Symposium applied Phytopathology: from the lab to the field, Wageningen	1 March, 2017
Wageningen PhD symposium: Local to Global, Wageningen	3 May, 2017
Symposium Solanaceae genetic resources in research and breeding, Nijmegen	26 April, 2017
Symposium on CRISPR-Cas - From Evolution to Revolution, Wageningen	8 March, 2018
► **Seminar plus**	
► **International symposia and congresses**	
8th European Plant Science Retreat in Barcelona, Spain	20 - 23 June, 2016
16th International Symposium on Insect-Plant relationships in Tours, France	2 - 6 July , 2017
Plant Biotic Stresses & Resistance Mechanisms in Vienna, Austria	2 - 3 July, 2018
► **Presentations**	
Green defence Against Pests (GAP) meeting (Oral)	21 April , 2015
Green defence Against Pests (GAP) meeting (Oral)	25 September, 2015
Green defence Against Pests (GAP) meeting (Oral)	6 April, 2016
8th European Plant Science Retreat in Barcelona, Spain (Poster)	20-23 June, 2016
Green defence Against Pests (GAP) meeting (Oral)	11 October, 2016
Green defence Against Pests (GAP) meeting (Oral)	18 May, 2017

16th International Symposium on Insect-Plant relationships in Tours, France (Poster)	2 - 6 July, 2017
Green defence Against Pests (GAP) meeting (Oral)	27 November, 2017
Green defence Against Pests (GAP) meeting (Oral)	13 March, 2018
3rd Plant Biotic Stresses & Resistance Mechanisms conference (Poster)	2-3 July, 2018
Green defence Against Pests (GAP) meeting (Oral)	21 September, 2018
Green defence Against Pests (GAP) meeting (Oral)	29 November, 2018
► **IAB interview**	
► **Excursions**	
EPS company visit - Enza Zaden, Enkhuizen	12 June, 2015
EPS company visit - TomatoWorld, Honselersdijk	14 October, 2016
Subtotal Scientific Exposure	**20.8**

3) In-Depth Studies	*date*
► **Advanced scientific courses & workshops**	
Genome Assembly, Wageningen	28-29 April, 2015
Introduction to R for Statistical Analysis, Wageningen	18 - 19 May, 2015
8th Utrecht International Summer school on Environmental Signaling, Utrecht	24-26 August, 2015
The Power of RNA-seq, Wageningen	10-12 Feb, 2016
Basic Statistics, Wageningen	14-23 December, 2016
Data Analyses and Visualizations in R	11-12 May, 2017
► **Journal club**	
PhD journal discussion group, Entomology (Twice a month)	2015-2018
Insect Plant Interactions discussion group, Entomology (Twice a month)	2015-2018
Molecular Ecology & Evolution discussion group, Entomology (Twice a month)	2017-2018
► **Individual research training**	
Subtotal In-Depth Studies	**8.0**

4) Personal Development	*date*
► **General skill training courses**	
EPS Introduction Course, Wageningen	22 September, 2015
Information Literacy PhD including Endnote Introduction, Wageningen	27-28 October, 2015
Wageningen Graduate Schools PhD Workshop Carousel, Wageningen	8 April, 2016
Data Management Planning, Wageningen	8 May, 2017
Efficient Writing Strategies 2, Wageningen	Oct - Dec, 2017
Last Stretch of the PhD Programme, Wageningen	8 December, 2017
► **Organisation of meetings, PhD courses or outreach activities**	
Organised PhD lunch bi-weekly meetings in Entomology, Wageningen	2015 - 2016
Organised EPS company visit to TomatoWorld	14 October, 2016
12th Plant-Insect Interaction Workshop, Wageningen	7 November, 2017
► **Membership of EPS PhD Council**	
EPS PhD council member	2016-2017
Subtotal Personal Development	**6.7**

TOTAL NUMBER OF CREDIT POINTS*	**37.0**

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

Appendix

The supplementary data of all chapters are available via the link below,

https://www.dropbox.com/sh/ae4su5khxozrglj/AAAj7VAG-Z14j8MfnC0o3SXga?dl=0

The data is organised chapter wise.

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