Transcription factors regulating terpene synthases in tomato trichomes

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Chapter 1

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1. Plant secondary metabolites

Plants produce a plethora of secondary metabolites- originally defined as such because their function was unclear, but they were known to be derived from the building blocks of primary metabolism like amino acids, lipids and sugars (Pichersky and Lewinsohn, 2011). However, given the accumulating evidence that secondary metabolites play important roles for example in the environmental adaptation of plants to both biotic and abiotic stress or in pollination (see sections 3 and 5), they have recently been termed “specialized” metabolites (Pichersky et al., 2006). Specialized metabolites can be divided into three main groups: phenolic compounds (phenylpropanoids, flavonoids), terpenoids/ isoprenoids and nitrogen-containing compounds (glucosinolates, alkaloids, cyanogenic glycosides) (Aharoni and Galili, 2011). A simplified scheme of the production pathways for some of these specialized metabolites is shown in Figure 1. Although different plant families can produce to some extend the same specialized metabolites, particular chemical classes are typically associated with a specific plant family- for example plants of the Solanaceae family are known to produce terpenoids, those of the Brassicaceae family accumulate glucosinolates, whereas Leguminous species produce typically flavonoids (Wu and Chappell, 2008).

Figure 1. Simplified scheme of specialized metabolite production pathways. Precursor compounds are in italics, major classes of specialized metabolites are in bold. Pyr; pyruvate, GA-3P; glyceraldehyde 3-phosphate, MVA; mevalonate, MEP; methylerthritol, PEP; phosphoenolpyruvate, E4P; erythrose 4-phosphate. Modified from Aharoni and Galili, 2011.
2. Isoprenoid biosynthesis

Plant isoprenoids (or terpenoids) constitute a large, structurally diverse class of chemical compounds, but the immediate precursors for all isoprenoid compounds are the simple C₅-units isopentenyl diphosphate (IPP) and dimethallyl diphosphate (DMAPP; Fig.2). IPP and DMAPP can be synthesized through the methylerythritol (MEP) pathway in the plastids. Alternatively IPP can be synthesized and then converted to DMAPP through the mevalonate (MVA) pathway in the cytosol (Bouvier et al., 2005). However, exchange of isoprenoid precursors between plastids and the cytosol has been reported (e.g. Dudareva et al., 2005). For a detailed description of the enzymatic steps involved in the MEP and MVA pathways, see Figure 2 in Chapter 5. Condensation of these C₅-units by prenyl diphosphate synthases leads to the formation of geranyl diphosphate (GPP) (or neryl diphosphate (NPP), the cis-isomer of GPP), farnesyl diphosphate (FPP) (or its cis-isomere (Z,Z)-FPP) and geranylgeranyl diphosphate (GGPP). Prenyl diphosphates are in turn converted to a variety of C₁₀ monoterpenes, C₁₅ sesquiterpenes and C₂₀ diterpenes by enzymes of the terpene synthase (TPS) family (Wu et al., 2006). Terpenes may be modified by hydroxylation, dehydration, decarboxylation or ring formation. Such modifications may involve the activity of cytochrome P450s that catalyze a wide variety of chemical reaction steps in plant secondary metabolism, ranging from hydroxylation to ring formation and carbon-carbon bond cleavage (Morant et al., 2003). In addition, terpenes can also be decorated with other molecules thus forming the more complex isoprenoids. Modifications and decorations of the parental mono-, sesqui- and di-terpenes ultimately result in the thousands of different terpenoid structures found in nature (Aharoni et al., 2006). Finally, it is worth mentioning that isoprenoid biosynthesis also takes place in the mitochondria where for example the ubiquinones are produced (Aharoni et al., 2005).
Figure 2. Simplified scheme of isoprenoid production pathways. Dashed lines indicate more than one enzymatic step. IPP; isopentenyl diphosphate, DMAPP; dimethallyl diphosphate, GPP; geranyl diphosphate, NPP; neryl diphosphate, FPP; farnesyl diphosphate, GGPP; geranylgeranyl diphosphate.

3. Roles of isoprenoids

Isoprenoids function in primary and specialized metabolism. In primary metabolism they serve various roles such as components of membranes (phytosterol), photosynthetic pigments (carotenoids and phytol side chain of chlorophyll) or hormones (gibberellins, brassinosteroids, abscisic acid), but the majority of plant isoprenoids function as specialized metabolites (Lange et al., 2000; Flores-Perez et al., 2010). These specialized metabolites are non-volatile or volatile chemicals that can be sequestered in the plant or emitted in the environment serving various roles (Langenheim, 1994). Non-volatile compounds can function as part of the plant’s direct defenses either by inhibiting or suppressing growth of pathogens- for example phytoalexins are well known for their antimicrobial activity (Ahuja et al., 2012), or by exhibiting toxic or deterrent effects against herbivores- for instance hydroxygeranyllinalool diterpene glycosides that are known to mediate resistance of Nicotiana attenuata plants.
against Manduca sexta (Heiling et al., 2010) or cucurbitacin C that is involved in spider mite resistance of cucumber (Balkema-Boomstra et al., 2003). Some volatile isoprenoids function as attractants of pollinators, seed dispersers and other beneficial animals, like the monoterpane linalool that is emitted from Clarkia breweri flowers (Pichersky et al., 1995). Many of the volatile isoprenoids however are emitted by herbivore-infested plants and serve as attractants of natural enemies (predators and parasitoids), thus providing indirect defense against herbivores. In this case these compounds can be released when feeding ruptures the structures in which they are stored (see section 4) or can be synthesized de novo at different time points after the onset of herbivore feeding (Pichersky and Gershenzon, 2002). Most of the volatile isoprenoids belong to the mono- and sesqui-terpene classes but also the C\textsubscript{16}-norterpane \((E,E)-4,8,12\)-trimethyltrideca-1,3,7,11-tetraene (TMTT) and its C\textsubscript{11}-analog \((E)-4,8\)-dimethyl-1,3,7-nonatriene (DMNT) are known to play a role in the plant’s indirect defenses (Ament et al., 2004; Lee et al., 2010). Induced terpene synthesis after wounding or herbivore attack has been reported from numerous plant species including tomato (van Schie et al., 2007), maize (Schnee et al., 2002; Schnee et al., 2006), poplar (Arimura et al., 2004a), lotus (Arimura et al., 2004b), cucumber (Mercke et al., 2004) and medicago (Gomez et al., 2005; Navia-Gine et al., 2009) and usually transcriptional activation of these terpene synthase genes correlates well with the emission of the terpene product (see section 5). Finally, volatile terpenes can play a role in plant-plant communication: when released from damaged plants they can induce defense responses in neighboring plants (Arimura et al., 2000).

4. Specialized metabolite production and storage sites

Plants have evolved dedicated structures for the production and storage of (volatile) specialized metabolites. Plant volatiles are usually lipophilic substances
with high vapor pressures and can be released from flowers, fruits, and vegetative tissue into the atmosphere, but also from the roots into the rhizosphere. In the flower petals, biosynthesis of plant volatiles takes place in specialized or nonspecialized epidermal and mesophyll cells, and their emission is in the vast majority tightly correlated with attraction of pollinators (Pichersky et al., 2006; Van Moerkercke et al., 2012). Also, roots contain secretory cells that can release volatiles, which play a role in the direct defense against microbial pathogens as well as in indirect defense, e.g., via the attraction of entomopathogenic nematodes (Rasmann et al., 2005; Wenke et al., 2010). Other common anatomical structures where plant specialized metabolites are stored include secretory cavities present in the skin of many fruits and special ducts, such as those found on evergreens, in which resins are stored in a mixture with volatile chemicals to keep the resin fluid but which can evaporate during exposure to air upon mechanical damage such that the resin hardens and seals the wound (Maffei, 2010).

However, especially well studied are the glandular trichomes, which can be found on vegetative tissues of many plant species and which are the source of various specialized metabolites (Tissier, 2012). Glandular trichomes are classified in different types according to their shape and structure. They can be divided into peltate and capitate trichomes like those found in the Lamiaceae and Asteraceae families: the peltate trichomes consist of one basal cell, one stalk cell, and many secretory cells (typically 4–18) while the capitate trichomes comprise a basal cell, a single or multicellular stalk, and a head consisting of one or two cells (Werker, 2000; Maffei, 2010). Tomato trichomes in particular can be categorized as one of seven types (Luckwill, 1943): for example the glandular trichomes (type VI) of the cultivated tomato (Solanum lycopersicum) consist of a stalk and a four-celled “head” (Fig.3). These four cells are small and have a large wall-less subcellular cavity on top in which specialized metabolites are stored (Simmons and Gurr, 2005). Plants can possess non-glandular trichomes as well (like the type III trichomes of cultivated tomato). These non-glandular trichomes in combination with the sticky exudates of the glandular trichomes constitute a mechanical barrier.
to the movement and feeding of herbivores and thus play a role in the direct defense of the plant (Channarayappa et al., 1992; Simmons and Gurr, 2005).

Primary metabolism in glandular trichomes is highly active to generate precursors for the specialized metabolic pathways on-site. Trichome cells of various plants, like for example Solanum species, contain chloroplasts, as well as a nucleus (Peterson and Vermeer, 1984; Pyke and Howells, 2002). Trichomes operate as a closed system with minimal import from the rest of the plant, usually being sucrose (Schilmiller et al., 2008). Cutin is often deposited in the wall of the lowest stalk cell of glandular trichomes in order to prevent the synthesized products to flow back into the plant (Fahn, 1988). Hence, trichome constituents, which can be autotoxic, are stored safely away from the other plant tissues in the subcuticular space of the gland cells. Finally, volatiles can be released when the head is ruptured by herbivore movement or be transported, actively or passively, out of the trichome into the air upon upregulation of their biosynthesis during indirect defenses (Gershenzon et al., 1992; Pichersky et al., 2006).

Figure 3. Cultivated tomato trichomes. (A) Glandular (type VI) and non-glandular (type III) trichomes found on a tomato stem (photograph by Jan van Arkel; IBED, UvA), (B) close-up of glandular trichomes consisting of a stalk and a four-celled “head” (from van Houten et al., 2012) and (C) 3D-reconstruction of multiple confocal images of a type VI trichome “head” to visualize autofluorescence (merge of GFP (emission 505-545nm) and RFP (emission 600-700nm) channels; images were obtained with a Zeiss LSM 510 confocal laser scanning microscope. Photograph by Erik Manders; SILS, UvA).
5. Examples of specialized metabolites involved in the plant defense system

Although the main focus of this thesis is volatile terpenes, comparisons will be made with other specialized metabolites and their biosynthetic pathways. Therefore examples are given here, not only for terpenes involved in plant defenses, but also for other classes of specialized metabolites.

The phenylpropanoid eugenol is synthesized from phenylalanine (derived from the shikimate pathway) that is converted in several enzymatic steps to coniferyl alcohol, an intermediate in the synthesis of lignins, and subsequently to coniferyl acetate that serves as substrate for the (iso-)eugenol synthase (I/EGS). IGS and EGS have been cloned from basil glands and petunia and Clarkia breweri flowers (Koeduka et al., 2006; Koeduka et al., 2008). Apart from the role of these compounds as floral attractants of pollinators, they also seem to serve in defense against herbivores as they have been shown to have larvicidal activity against Spodoptera litura (Bhardwaj et al., 2010) and insecticidal activity against Tribolium castaneum and Sitophilus zeamais (Huang et al., 2002).

The fatty acid derivatives C_{11}, C_{13}, C_{15} methylketones are synthesized in high abundance in glandular trichomes of the wild tomato specie Solanum habrochaites glabratum, consisting mostly of 2-tridecanone and 2-undecanone (Antonious, 2001). They are produced in the plastids through the action of methylketone synthase 2 (ShMKs2) that hydrolyzes intermediates of fatty acid biosynthesis 3-ketoacyl-acyl carrier proteins, thus releasing 3-ketoacids. Subsequent decarboxylation by ShMKs1 of those 3-ketoacids leads to the formation of the methylketone products (Fridman et al., 2005; Ben-Israel et al., 2009; Yu et al., 2010). Direct toxicity of methylketones has been observed against spider mites (Chatzivasileiadis and Sabelis, 1997).

There are several terpene compounds from various plant species that have been shown to play a role in the defense system either individually or as part of a volatile blend released by the plants and a few examples are presented here.
However it must be noted that not for all terpene products, the respective terpene synthase has been identified.

Linalool, synthesized in tomato glandular trichomes through the activity of the monoterpene synthase 1 (*SlMTS1*), has been shown to be emitted after wounding, in correlation with the increased transcript abundance after spider mite feeding or artificial wounding (van Schie et al., 2007). Furthermore the tomato monoterpenes *p*-cymene, γ-terpinene, β-phellandrene and α-myrcene, as well as the sesquiterpene zingiberene and its conversion product curcumene were shown to exert a repellent effect on whiteflies (Bleeker et al., 2009). Except for *p*-cymene, terpene synthases that produce these monoterpenes either as a single compound or as a mixture with others have been identified (for overview: Falara et al., 2011; Chapter 5) and recently the tomato terpene synthase responsible for the synthesis of zingiberene has been identified (Bleeker et al., submitted). Maize plants upon herbivory by lepidopteran larvae emit a mixture of compounds that attracts females of the parasitic wasp *Cotesia marginiventris*. This volatile bouquet is produced by a single terpene synthase (TPS10), which is induced in herbivore-damaged leaves, and produces various sesquiterpenes, but mainly (*E*)-β-farnesene and (*E*)-α-bergamotene (Schnee et al., 2006). In medicago, (*E*)-β-ocimene is constitutively produced in leaves by *MtEBOS* at low levels, but *Spodoptera exigua* feeding up-regulates its production (Navia-Gine et al., 2009). Finally, metabolite analysis of cucumber leaves with and without spider mite infestation lead to the identification of DMNT (deriving from 3S-(*E*)-nerolidol), (*E*)-β-ocimene and (*E*,*E*)-α-farnesene as the three major terpenes emitted from infested cucumber plants. Furthermore, transcriptome analysis of the same samples lead to identification of CsaαFS, the enzyme responsible for the formation of (*E*,*E*)-α-farnesene from FPP. A 3S-(*E*)-nerolidol and (*E*)-β-ocimene synthase were not identified, however CsaαFS could produce (*E*)-β-ocimene from GPP suggesting a dual role for this enzyme (Mercke et al., 2004).
6. Regulation of isoprenoid biosynthesis

Plant defenses are costly and require resources otherwise used for growth and reproduction (Walters and Heil, 2007). Therefore, plants have evolved a complex, largely hormonal, signaling network to arrange defense and resource allocation (Pieterse et al., 2009). As it has been indicated in section 5, regulation of induced terpene biosynthesis occurs mostly at the transcript level of the terpene synthases, as well as on transcriptional regulation of precursor genes (discussed in Chapter 5), but also of downstream modifying enzymes, like the cytochrome P450s (Son et al., 1998; Luo et al., 2001). Activation of the specialized metabolite pathways in response to a stress signal is often triggered by phytohormones like jasmonic acid, ethylene, salicylic acid or abscisic acid (Nascimento and Fett-Neto, 2010).

6.1 Regulation of plant defenses at the gene expression level

The general principle for the activation of plant specialized metabolite biosynthesis involves a signal that initiates a transduction cascade leading to de novo biosynthesis or activation of transcription factors that in turn regulate the expression of specialized metabolite biosynthetic genes (Zhao et al., 2005). Regulation of gene expression in all eukaryotes involves sequences that flank the gene in question containing cis-regulatory elements (promoters) and proteins that recognize and can interact with these elements (trans-acting factors; Wray et al., 2003).

6.1.1 Trans-acting factors and cis-elements

Trans-acting factors (transcription factors; TFs) are DNA-binding proteins that can recognize and bind specific regulatory sequences, the so-called cis-elements, in the
promoter of target genes and thus affect the rate of transcription initiation (Wray et al., 2003). Cis-elements are typically located adjacent to the promoter, but can also be found far upstream of it, in introns or even downstream of a gene, but DNA looping allows the gene regulatory proteins bound at any of these positions to interact with the proteins that assemble at the core promoter (Wray et al., 2003). In general, activating transcription of a gene involves decondensation of chromatin around the core promoter and some TF binding site(s), followed by the binding of general TFs that recruit the RNA polymerase II complex onto the basal promoter (Lee and Young, 2000). Apart from a DNA-binding domain, TFs usually have a transcription regulation domain (activating or repressing; Liu et al., 1999). Repressors inhibit transcription through various mechanisms, including competing with an activator for the DNA binding site, modifying chromatin structure or preventing recruitment of the transcription initiation complex (Lee and Young, 2000).

Control of a cellular process (like response to a stress stimulus) requires the coordinate activation or repression of genes in an exact spatial and temporal pattern and such regulation can be achieved by interaction of a TF with specific cis-elements of the target gene(s) and by temporal and spatial expression of transcription factors themselves. Therefore, even TFs that share DNA-binding properties can control distinct biological processes (de Folter and Angenent, 2006). Such transcriptional regulation of TFs by upstream genes is part of the cells’ regulatory network that will not be discussed here.

### 6.1.2 Transcription factor families

Transcription factors can be classified into different families according to their DNA binding domain. There are at least 64 TF families found in vascular plant genomes (Rushton et al., 2008) and binding of these proteins to the DNA bases commonly takes place through the secondary structure elements of a β-sheet or an
The cis-elements TFs recognize and bind to are usually short stretches of 4-8 base pairs and with a few variations from a consensus can be characteristic for each TF family. However, specificity for the target promoters can partly be determined by adjacent sequences (Rushton et al., 2010; Figueroa et al., 2010).

The first plant TF identified was a MYB domain protein required for the synthesis of anthocyanins in maize kernels (Paz-Ares et al., 1987). MYB TFs are involved in the regulation of an array of processes including secondary metabolism (e.g. flavonoid biosynthesis), cell fate and identity (e.g. trichome formation), development (e.g. anther development) or abiotic and biotic stress responses (e.g. drought stress and disease resistance; Dubos et al., 2010). DNA-binding specificity varies among different MYB proteins within and between plant species and only few DNA binding sites have been characterized functionally (Martin and Paz-Ares, 1997; Dubos et al., 2010). For example R2R3-MYB family members of group A bind to a CNGTT(A/G) motif (Prouse and Campbell, 2012).

Another big family of plant TFs took its name from its founding member Apetala 2 that controls Arabidopsis flower and seed development (Jofuku et al., 1994). AP2 domain proteins are involved in regulating developmental processes as well as in abiotic stress acclimation and hormone-dependent signaling in response to, for example pathogens (Dietz et al., 2010). Members of the subgroup AP2/ERF have been implicated in defense responses mediated by jasmonates (see section 6.2) in a number of plant species (Rushton et al., 2008) including Catharanthus roseus (discussed in section 6.4). There are four subgroups in the family, each recognizing different core elements (for example the dehydration-responsive element (DRE) by the DREB subfamily or the GCC box (GCCGCC) by the ERF subfamily; Dietz et al., 2010).

Basic helix-loop-helix (bHLH) transcription factors bind DNA through their N-terminal basic region, whereas the C-terminal HLH region functions as a dimerization domain (Toledo-Ortiz et al., 2003). They form one of the largest families of TFs in plants, however only a relative small number of bHLH genes
have been studied in detail, some of which are involved in processes like anthocyanin biosynthesis, trichome differentiation or light signaling (Heim et al., 2003). These proteins typically bind to an E box motif (CANNTG) with, for example, MYC bHLH proteins recognizing a variation of the E box, known as the G box motif (CACGTG; Heim et al., 2003). Roles of Arabidopsis, Nicotiana tabacum and C. roseus MYC transcription factors are presented in following sections.

Zinc finger (ZnF) transcription factors comprise different classes of TFs that all involve a sequence motif in which a zinc atom is bound by cysteine (C) and histidine (H) residues stabilizing the secondary structure of the “finger” (Klug, 2010). The zinc finger domain however, enables different proteins to bind not only DNA, but also bind or interact with RNA or other proteins (Ciftci-Yilmaz and Mittler, 2008). There are different classes of ZnF TFs such as the C2H2-type, DNA binding with one finger (Dof), WRKY and RING-finger (discussed in Chapter 4) that play a role in regulating various processes (Takatsuji, 1998). Consensus binding site for example the WRKY TFs is the W box (TTGACC/T) for almost all proteins investigated so far (Rushton et al., 2010).

An overview of all transcription factor families and their characterized members from various plant species can be found in publicly available TF databases. Such databases facilitate comparative studies of transcriptional regulation in model plants like Arabidopsis (Guo et al., 2005), tobacco (Rushton et al., 2008), rice (Gao et al., 2006), poplar (Zhu et al., 2007) or 46 other plant species (Zhang et al., 2011a).

6.2 Role of hormones in plant defenses

Whereas plant resistance against immobile pathogens often is characterized by a hypersensitive response (HR), defense against herbivores is associated more with a decrease in tissue palatability (Anten and Pierik, 2010). Central in the organization
of anti-herbivore defenses is the plant hormone jasmonic acid (JA) and its active
derivative JA-isoleucine (JA-Ile), which rapidly accumulates during herbivory. The
mode of action of JA has been studied in detail using JA biosynthesis- or
perception-impaired mutant plants, which are often preferred by herbivores in
choice tests while allowing for higher herbivore fitness (Howe and Jander, 2008).
Accumulation of JA-dependent defense proteins and metabolites is often co-
regulated by ethylene in a synergistic manner. In contrast, salicylic acid (SA)
antagonizes the action of JA (Pieterse et al., 2009). SA is well known for its
signaling role in defenses induced by biotrophic pathogens, but many stylet-
feeding herbivores, like mites, whiteflies, and aphids, induce a cocktail of JA- and
SA-related responses (Kant et al., 2008). Although it is not clear to which extent
this mixed response is required for the plant to establish the appropriate defenses,
the “decoy hypothesis” suggests that in some cases, the herbivore could benefit
from a SA-mediated suppression of the JA defenses (Zarate et al., 2007). Finally,
also the hormones auxin and abscisic acid (ABA) influence the properties of the
signaling network mostly via antagonizing the action of JA and SA (Pieterse et al.,
2009). The dynamics of this complex regulatory network, in which hormonal
synergisms and antagonisms determine the final output of the defense response,
depend largely on the type of herbivore as well as on the physiological status of the
plant.

Since herbivory and wounding mainly elicit responses mediated by jasmonic acid
as part of the plants’ (in)direct defenses, the focus in the next section will be on this
hormone.

6.2.1 Jasmonic acid biosynthesis and regulation of downstream genes

The precursor for the biosynthesis of JA is α-linolenic acid released by lipases
from chloroplast galactolipids. Activity of 13-lipoxygenase (LOX), allene oxide
synthase (AOS) and allene oxide cyclase (AOC) enzymes converts α-linolenic acid to cis-(+)-12-oxophytodienoic acid (OPDA). In a parallel pathway dinor-OPDA can be formed from hexadecatrienoic acid by the same set of enzymes. OPDA (and dinor-OPDA) is translocated into the peroxisomes where OPDA reductase 3 (OPR3) catalyzes its reduction to oxo-pentenyl-cycloheptan-octanoic acid (OPC-8) that in turn undergoes three rounds of β-oxidation leading to jasmonyl-CoA (JA-CoA) formation. JA-CoA is then cleaved by a putative thioesterase yielding (+)-7-iso-JA that equilibrates to the more stable (-)-JA (Wasternack and Kombrink, 2010).

In an uninduced situation transcription factors (TFs) are bound to the target elements in the promoters of JA-responsive genes, but their transcriptional activity is repressed by the binding of a repressor complex that in Arabidopsis consists of Jasmonate ZIM domain (JAZ), Novel Interactor of JAZ (NINJA) and Topless (TPL; Pauwels et al., 2010). There are 12 members of the JAZ family in Arabidopsis with functional redundancy, but also diverse tissue- and stage-specific expression patterns (Chini et al., 2009) and homologues are found in many plant species (Chico et al., 2008). In general, upon perception of a stress stimulus (-)-JA is converted to its active form JA-isoleucine (JA-Ile) through the action of JA amino acid conjugate synthase 1 (JAR1). High levels of JA-Ile lead to binding by the jasmonate receptor Coronatine Insensitive 1 (COI1) that is part of the Skp1/Cullin/F-box (SCF) complex and acts as an E3 ubiquitin ligase. The SCF COI1 complex recruits the JAZ proteins that get polyubiquitinated and are directed to the 26S proteasome for degradation, releasing the repression of the TFs they were bound to, and thus activating expression of the downstream JA-responsive genes. Among the early JA-responsive genes are those encoding JAZ proteins, creating a negative feedback loop (Wasternack and Kombrink, 2010). Similar roles for COI1 homologues have been reported in other plant species, including tomato (Li et al., 2004) and the image emerges that a JAZ-mediated signaling web regulates JA-dependent functions, like specialized metabolite production or responses to abiotic and in addition developmental cues. In tomato, jasmonic acid-insensitive 1 (jai1;
the homolog of the Arabidopsis COI1 gene) plants exhibited abnormal development of glandular trichomes and defense-related phenotypes like severely compromised resistance to spider mites (Li et al., 2004). The wound-induced systemic defense in tomato is believed to start with the interaction of systemin, an 18 amino acid peptide cleaved from the C-terminal region of the 200 amino acid precursor prosystemin, with a receptor in the plasma membrane, activating JA biosynthesis (Sun et al., 2011) in a cascade of events like those described above for Arabidopsis. However, in tomato no targets of JAZ proteins have been identified. AtMYC2 was until recently the only transcription factor described as a direct JAZ target (Chini et al., 2007), but two closely related bHLH TFs, AtMYC3 and AtMYC4 were shown to act additively with MYC2 in the activation of JA responses in Arabidopsis- i.e. playing a role in root growth and regulation of herbivory and pathogen responses (Fernandez-Calvo et al., 2011). In other plant species similar roles for JAZ and MYC proteins have recently been shown. For example in Nicotiana tabacum the biosynthesis of the alkaloid nicotine involves the action of NtCOI1, NtJAZ and NtMYC2 (Shoji and Hashimoto, 2011; De Boer et al., 2011; Zhang et al., 2011b) and in rice drought tolerance is conferred through a OsCOI1, OsJAZ and OsbHLH148 mediated cascade (Seo et al., 2011).

6.3 Additional modes of regulation

There are additional levels on which gene expression can be regulated, which will be briefly presented here. Posttranscriptional regulation is known to be an important mechanism defining final concentration of the active gene product and it includes control of RNA (alternative) splicing, mRNA stability, or miRNA-mediated degradation (Blencowe et al., 2009). Additionally, protein-protein interactions or posttranslational regulation can significantly impact the regulatory activity of transcription factors. Transcriptional activity of for example some MYB TFs depends in vivo on protein-protein interactions, with homo- or hetero-
dimerizations enabling DNA recognition with high affinity and specificity (Dubos et al., 2010). Posttranslational modifications of proteins include acetylation, hydroxylation, ubiquitination and more commonly phosphorylation, and such modifications can influence among others DNA binding affinity or protein stability by altering for example protein conformation (Vom Endt et al., 2002). Finally, epigenetic regulation (histone modifications and DNA methylation) can play a role in chromatin structure and so determine the transcriptional state and expression level of genes (Chinnusamy and Zhu, 2009).

6.4 Examples of transcription factors regulating terpenoid biosynthesis

There are relatively few transcription factors that regulate terpenoid biosynthesis identified to date, which are presented here. For an overview of TFs regulating (JA-induced) secondary metabolite biosynthesis see De Geyter et al., 2012.

The sesquiterpene phytoalexin gossypol is produced in the glands of cotton (Gossypium arboreum) aerial tissues from the biosynthetic precursor (+)-δ-cadiene. CAD1 ((+)-δ-cadiene synthase) catalyzes the first committed step towards the formation of gossypol and analysis of its promoter sequence revealed the presence of two W box cis-acting elements forming a palindrome (Xu et al., 2004). Degenerate primers were used to isolate WRKY cDNAs from a cotton cDNA library, but only one of the 10 fragments isolated had higher transcript levels in a glanded cotton cultivar than in a glandless cultivar. This cDNA was cloned full-length and designated GaWRKY1. CAD1 and GaWRKY1 had a similar spatial and temporal expression pattern. Furthermore, elicitor and jasmonate treatments induced transcription of both genes. GaWRKY1 was shown to localize to the nucleus and could bind to three tandem repeats of the W box palindrome of CAD1 in yeast and in vitro. Finally, in transgenic Arabidopsis plants it was shown that
overexpression of *GaWRKY1* strongly activated the *CAD1* promoter (Xu *et al.*, 2004).

The sesquiterpene lactone endoperoxide artemisinin is produced in glandular trichomes of *Artemisia annua* with the first committed step for its biosynthesis being the cyclization of FPP by the amorpha-4,11-diene synthase (ADS). Analysis of the *ADS* promoter sequence revealed the presence of two reversely oriented W box *cis*-acting elements (Ma *et al.*, 2009). A WRKY EST was identified in a glandular trichome cDNA library and was designated AaWRKY1. *ADS* showed the same expression pattern as *AaWRKY1* and both were induced by elicitor and jasmonate treatments. Furthermore, AaWRKY1 was shown to localize to the nucleus and could bind to three tandem repeats of the W box of *ADS* in yeast and *in vitro*. Co-expression of 35S:AaWRKY1 and ADSp:GUS in stably double-transformed tobacco plants strongly activated GUS expression and finally transient overexpression of AaWRKY1 in *A. annua* leaves was shown to clearly activate expression of *ADS*, but also of the MVA pathway precursor 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*) as well as of the downstream genes cytochrome P450 reductase (*CPR*) and artemisinic aldehyde reductase (*DBR2*). Notably *FPS* expression was not upregulated suggesting that FPS is not a rate-limiting factor in artemisin biosynthesis (Ma *et al.*, 2009).

The *Nicotiana attenuata* *WRKY3* and *WRKY6* were strongly induced in response to wounding and oral secretions (OS) of *Manduca sexta* caterpillars, respectively (Skibbe *et al.*, 2008). However, neither of these TFs was transcriptionally induced by JA. It was proposed that they function upstream of the JA signal, since silencing *WRKY3* and/or *WRKY6* lead to reduced expression of JA-biosynthetic genes, as well as reduced levels of JA. Silenced plants were more vulnerable to herbivore feeding, which was associated with reduction in direct defense-related Trypsin Proteinase Inhibitors (TPI) protein activity as well as reduced emission of the indirect defense sesquiterpene *cis*-α-bergamotene. Exogenous application of JA-supplemented OS to the WRKY-silenced plants restored TPI activity levels and the amount of *cis*-α-bergamotene released (Skibbe *et al.*, 2008).
The common precursor for the synthesis of a range of terpenoid indole alkaloids (TIA) in *Catharanthus roseus* is 3α-(S)-strictosidine. Strictosidine is formed by condensation of secologanin (its terpenoid part, produced via multiple enzymatic conversions of the MEP pathway-derived monoterpenoid geraniol) with tryptamine (the indole moiety, produced via the shikimate pathway), through the action of the enzyme strictosidine synthase (*STR*; Fig. 4). Tryptamine is formed by decarboxylation of tryptophan by the enzyme tryptophan decarboxylase (*TDC*; Memelink *et al*., 2001). A yeast-one-hybrid (Y1H) screen with the JA- and elicitor-responsive element (JERE) of the *STR* promoter led to the identification of two cDNAs encoding AP2/ERF domain proteins that were designated octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA) 1 and 2. However only *ORCA2* mRNA levels were induced by jasmonate and elicitor treatments (Menke *et al*., 1999). A genetic (T-DNA activation tagging) approach identified another AP2/ERF domain-containing TF that was named ORCA3. Overexpression of *ORCA3* in transgenic *C. roseus* cell cultures induced primary (like 1-deoxy-D-xylulose 5-phosphate synthase; *DXS*) and secondary (like *STR* and *TDC*) metabolic pathway genes that are involved in TIA biosynthesis, all of which were shown to be methyl-jasmonate (MeJA)-inducible. Furthermore, upon addition of excess terpenoid precursor (loganin), TIA production was increased in the *ORCA3* overexpressing cell lines (van der Fits and Memelink, 2000). Like ORCA2, ORCA3 was shown to specifically bind to the JERE fragment of the *STR* promoter. Moreover, treatment of *C. roseus* cell cultures with MeJA and/or a protein synthesis inhibitor (cycloheximide; CHX) lead to the discovery that *ORCA3* induction by MeJA does not depend on *de novo* protein synthesis, but also that MeJA-induced *STR* and *TDC* expression does not require *de novo* synthesis of ORCA3 (or other TFs), therefore jasmonate most likely induces *STR* and *TDC* expression via modification (possibly phosphorylation) of pre-existing ORCA3 protein (van der Fits and Memelink, 2001). Expression of *ORCA3* is itself rapidly induced by MeJA, which implies either autoregulation or regulation by upstream TF(s). By analyzing the *ORCA3* promoter, a jasmonate-responsive element (JRE)
was identified that consists of an A/T-rich quantitative sequence determining expression level and a G box-like qualitative sequence, likely to bind a bHLH TF, responsive to MeJA. Y1H screens identified (non MeJA-inducible) clones containing an AT-hook motif that could bind to the quantitative sequence, but none that could bind the qualitative sequence (Vom Endt et al., 2007). *C. roseus* bHLH MYC cDNA clones had been previously identified in a Y1H screen with a tetramer of the *STR* promoter G box (located upstream of the JERE element; Pre et al., 2000), and the one with highest homology to AtMYC2 was designated CrMYC2 and shown to be strongly induced by MeJA and that it could bind to the G box-like qualitative sequence of the *ORCA3* promoter *in vitro* and *in vivo*. Surprisingly, although CrMYC2 could bind to the G box from the *STR* promoter, it could not activate gene expression via native *STR* promoter derivatives containing this same G box. CrMYC2 knock down lines showed strong reduction in the levels of MeJA-responsive *ORCA2* and *ORCA3* mRNA as well as alkaloid accumulation, but no effect on *STR* and *TDC* expression (Zhang et al., 2011c). Since MeJA induction of *ORCA3* and CrMYC2 was equally fast and *ORCA3* induction is insensitive to CHX (as is the CrMYC2 induction), it is probably not dependent on *de novo* CrMYC2 protein synthesis, but caused by activation of pre-existing CrMYC2 protein. This activation could involve degradation of JAZ proteins that, in analogy with the *Arabidopsis* system, repress CrMYC2 activity in the absence of MeJA (Zhang et al., 2011c).

Additional complexity to the regulation of jasmonate-induced TIA biosynthesis in *C. roseus* brings the identification of three more classes of TFs involved in the process. The Y1H screen with a tetramere of the *STR* promoter G box allowed the identification of CrMYC TFs, but also of two TFs of the basic leucine zipper (bZIP) class (Pre et al., 2000), designated as G box-binding factors (CrGBF) 1 and 2, which were shown to be repressors of the *STR* gene expression via direct interaction with the G box (Siberil et al., 2001). Furthermore, Y1H screens of the elicitor-responsive element of the *TDC* promoter led to the isolation of three C2H2-type ZnF TFs that were designated zinc finger *Catharanthus* transcription factors.
(ZCT) 1, 2 and 3 and were shown to act as transcriptional repressors of \textit{TDC} and \textit{STR} promoter activity. Furthermore it was shown that yeast elicitor and MeJA increased \textit{ZCT} mRNA levels and, interestingly, that the \textit{ZCT} proteins could repress the activating activity of ORCA 2 and 3 on the \textit{STR} promoter without competing for the same binding sites (Pauw et al., 2004). Finally, analysis of available promoters from the TIA pathway genes identified W box(es) in almost all of them and therefore degenerate primers were designed on conserved sequences of WRKRY domains in order to identify mRNAs in MeJA-treated \textit{C. roseus} tissues. One of the open reading frames obtained, designated CrWRKY1, was shown to be induced by MeJA and could bind to the \textit{TDC} promoter sequence containing the W box \textit{in vitro} and in yeast. Overexpression of CrWRKY1 resulted in much higher \textit{TDC}, but also moderately higher \textit{ZCT} levels. However, this strongly reduced the expression of transcriptional activators \textit{ORCA2}, \textit{ORCA3} and to a lesser extend \textit{CrMYC2} and had no effect on \textit{STR} expression. Furthermore, these overexpressing transgenic lines showed accumulation of TIA serpentine in the roots, where \textit{CrWRKY1} is highest expressed (Suttipanta et al., 2011).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{regulatory_network}
\caption{Regulatory network of terpene indole alkaloid (TIA) biosynthesis in \textit{Catharanthus roseus}. Only key players mentioned in the text are shown here. Pathway enzymes are in boxes, transcription factors are in ovals. Dashed lines indicate more than one enzymatic step. Arrows indicate positive interactions, T-bars indicate negative interactions. For abbreviations see text.}
\end{figure}
It becomes clear from the well-studied case of terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* and what has been discussed so far, that regulators can form a dynamic network that controls the temporal- and tissue-specific expression of genes, with cross-talk between transcription factors with overlapping functions and (concurrent) induction of activators and repressors by elicitors and stress or developmental signals.

7. Thesis Outline

Chapter 1 gave a general introduction to isoprenoids and terpenes, how and where they are synthesized, the roles terpenes play in plants and how terpene biosynthesis is regulated. In Chapter 2, an overview of the terpene synthase family in tomato is given and a subset of mono- and sesqui-terpenes are further characterized, with a focus on genes that are expressed in the trichomes. Chapter 3 describes the dissection of the promoter of tomato monoterpene synthase 1 (*SlMTS1*) and the identification of a putative transcription factor that binds to a 207bp fragment proximal to the coding region. This TF was designated Emission of Terpenes 1 (*SlEOT1*) and further characterized in Chapter 4. In Chapter 5 an analysis of transcriptomic data from tomato stem trichomes is presented. Two transcription factors from this EST dataset are shown to interact with terpene synthase (described in Chapter 2) promoters in a transient assay *in planta*. Finally, in Chapter 6 results presented in this thesis are collectively discussed.
8. Account


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