MYC transcription factors: masters in the regulation of jasmonate biosynthesis in Arabidopsis thaliana

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Chapter1

General Introduction

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In nature, plants are continuously challenged by a myriad of environmental changes that comprise various biotic and abiotic stresses, such as those brought about by microbial pathogens, herbivorous insects, wounding, drought or salinity. Because plants have a sessile lifestyle, they have developed sensitive sensory systems to perceive external attacks and sophisticated strategies to mount effective responses to defend themselves against all these different types of stresses. Optimal plant fitness in the face of those threats relies on complex signal transduction networks that link damage-associated signals to appropriate changes in metabolism, growth, and development. Plant hormones, a group of structurally diverse small molecules, act as central players in the plant defensive signaling network and are key regulators of plant growth and development (Bari and Jones, 2009; Santner and Estelle, 2009). Attack or environmental stimuli result in changes in the levels of the major defense hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). Other plant hormones, including abscisic acid (ABA), gibberellic acids (GAs), auxin and cytokinins, are also implicated in plant defense signaling pathways (Bari and Jones, 2009; Pieterse et al., 2012).

Jasmonates (JAs), consisting of JA and its cyclic precursors and derivatives, act as important molecules in many developmental processes and in defense against environmental stresses. JAs modulate diverse processes such as vegetative growth (Staswick et al., 1992), fruit ripening (Kondo et al., 2000), trichome formation (Traw and Bergelson, 2003) and flower development (Browse, 2009). Plant responses to wounding, insect herbivory and necrotrophic pathogens are orchestrated by JAs (Yan et al., 2013). In the past decades, JAs biosynthesis, perception, signal transduction and action in Arabidopsis have been widely investigated and extensively reviewed.

JAs biosynthesis

JAs are plant oxylipins and are synthesized via the octadecanoid pathway. Most of the enzymes of this pathway leading to JAs biosynthesis and metabolism have been identified and several reports have reviewed their structures, biochemical activities and functional regulation (Schaller, 2001; Creelman and Mulpuri, 2002; Schaller and Stintzi, 2009; Yan et al., 2013). The biosynthesis of JAs begins with the release of α-linolenic acid (α-LeA) from chloroplast membranes by the action of a lipid hydrolyzing enzyme. Upon α-LeA liberation in the chloroplast, this fatty acid is then converted by 13-lipoxygenase (13-LOX) to 13-hydroperoxylinolenic acid (13-HPOT),

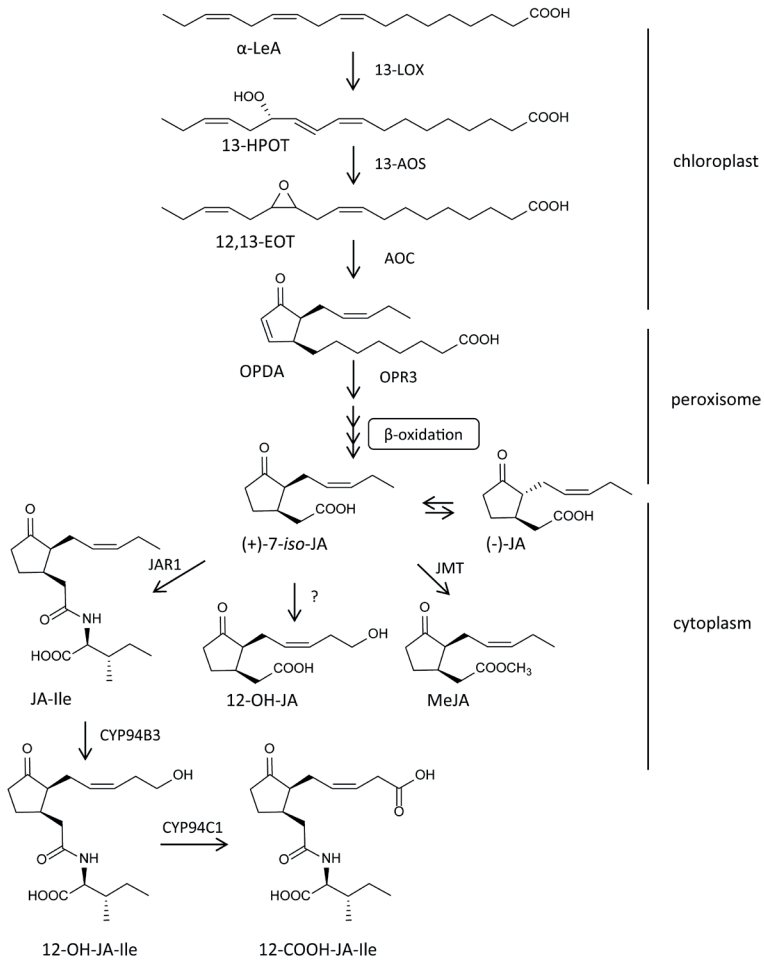

Figure 1. Scheme of the JAs biosynthesis pathway in *Arabidopsis thaliana*. The enzymes and the intermediates are indicated as 13-LOX for 13-lipoxygenase, 13-AOS for 13-allene oxide synthase, AOC for allene oxide cyclase, OPR3 for OPDA reductase, JAR1 for jasmonate resistant 1, JMT for JA carboxyl methyltransferase; α-LeA for α-linolenic acid, 13-HPOT for 13-hydroperoxylinolenic acid, 12,13-EOT for 12,13-epoxyoctadecatrienoic acid, OPDA for 12-oxo-phytodienoic acid, (+)-7-*iso*-JA and (-)-JA for jasmonic acid, JA-Ile for jasmonoyl-L-isoleucine, 12-OH-JA for 12-hydroxyjasmonic acid, MeJA for methyl jasmonate, 12-OH-JA-Ile for 12-hydroxy-JA-Ile and 12-COOH-JA-Ile for oxidized 12-hydroxy-JA-Ile.

which is the substrate for 13-allene oxide synthase (13-AOS). 13-AOS catalyzes the conversion of 13-HPOT to the unstable epoxide 12,13-epoxyoctadecatrienoid (12,13-EOT), providing substrate for the following enzyme allene oxide cyclase (AOC) and resulting in the formation of 12-oxo-phytodienoic acid (OPDA). OPDA is subsequently transported from chloroplasts to peroxisomes where it is reduced

by OPDA reductase (OPR) to 3-oxo-2-(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0). Three rounds of β-oxidation reactions result in the formation of (+)-7-*iso*-JA, which readily isomerizes to the thermodynamically favored (-)-JA which is the predominant form of JA in plant tissues (Schaller et al., 2004). In addition to isomerization, JA can undergo different molecular modifications to produce a variety of derivatives in plants, among which jasmonoyl-L-isoleucine (JA-Ile) is considered as the main bioactive JAs (Fonseca et al., 2009). JA-Ile is synthesized by a JA amino acid synthetase (JAR1) that conjugates (+)-7-*iso*-JA to isoleucine (Suza and Staswick, 2008). Another well-characterized modification is the methylation to methyl-JA (MeJA) by JA carboxyl methyltransferase (JMT) (Seo et al., 2001). The cytochrome P450 enzyme CYP94B3 was found to hydroxylate bioactive JA-Ile to the inactive compound 12-hydroxy-JA-Ile (12-OH-JA-Ile) and the P450 enzyme CYP94C1 was found to act in the subsequent carboxylation step leading to the inactive compound 12-COOH-JA-Ile (Heitz et al., 2012). Together with hydroxylation of JA to 12-OH-JA (Miersch et al., 2008), hydroxylation and carboxylation of JA-Ile are assumed to be 'switch off' mechanisms in JAs signaling (Wasternack and Hause, 2013).

Perception of external stresses triggers the biosynthesis of JAs for the adaptation of plants to the changing environment. Leaf damage inflicted by mechanical wounding and herbivory are highly effective triggers for *de novo* JAs synthesis and result in rapid increases in JAs accumulation at the site of wounding (Glauser et al., 2008). The Arabidopsis *fad3fad7fad8* triple mutant, which is JAs deficient and exhibits enhanced susceptibility to insect attack (McConn et al., 1997), provides proof that *de novo* JAs synthesis is required to protect plants against attack by certain insects. The rapid production of JAs results from the sufficient occurrence of JAs biosynthesis enzymes and the release of substrates from membranes upon strong external stimuli such as wounding (Stenzel et al., 2003). Many studies have shown that most biosynthesis genes including *LOX*, *AOS*, *AOC*, *OPR3*, *JMT*, and *JAR1* are induced by JAs treatment and wounding (Sasaki et al., 2001; Schaller, 2001; Wasternack, 2007). It is widely accepted that JAs biosynthesis is regulated by a positive feedback loop. However, constitutive overexpression of AOS and AOC did not alter basal JAs levels (Laudert et al., 2000; Stenzel et al., 2003a). Surprisingly, JAs biosynthesis is not induced by endogenous JAs in tomato leaves (Miersch and Wasternack, 2000) and the accumulation of JAs is undetectable in response to JA although JAs biosynthesis genes are expressed (Scholz et al., 2015), suggesting additional post-translational regulation mechanisms. Furthermore, the abundant appearance

of LOX, AOC and AOS proteins in fully developed leaves is independent of stress-induced accumulation of the corresponding mRNAs (Stenzel et al., 2003b). Therefore, it has been suggested that substrate availability, enzyme activity and tissue specificity are important factors in JAs biosynthesis besides gene transcription (Scholz et al., 2015; Wasternack, 2007).

JAs perception and signaling pathway

Following the production of JAs, transduction of the JAs signal occurs via interaction with a receptor that binds bioactive JA-Ile. In order to discover JAs receptor proteins, screening for Arabidopsis mutants insensitive to growth inhibition by MeJA or coronatine (a functional and structural analog of JA-Ile) was performed (Staswick et al., 1992; Feys et al., 1994). Exhaustive screens identified that the *coronatine insensitive1* (*coi1*) mutant was insensitive to JAs, male sterile, defective in resistance to certain insects and pathogens and failed to express JAs-related genes (Benedetti et al., 1995; McConn et al., 1997; Thomma et al., 1998). The Arabidopsis *COI1* gene was found to encode an F-box protein (Xie et al., 1998). F-box proteins were known to associate with the proteins Skp, Cullin and Rbx to form an SCF complex with E3 ubiquitin ligase activity (Bai et al., 1996). Co-immunoprecipitation experiments confirmed that COI1 associated physically with SKP1, CUL1 and Rbx1 proteins *in vivo* to assemble the SCF^{COI1} complex (Devoto et al., 2002; Xu et al., 2002). Furthermore, additional mutants that were deficient in other components or regulators of SCF complexes also showed impaired JAs responses (Tiryaki and Staswick, 2002; Feng et al., 2003; Lorenzo and Solano, 2005), further supporting the notion that the core JAs signaling module and JAs responses depend on the actions of the SCF^{COI1} complex. These discoveries led to the suggestion that JAs signaling involves the ubiquitination of specific proteins by the SCF^{COI1} complex and their subsequent degradation by the 26 proteasome (Turner et al., 2002). According to this hypothesis, the substrates that COI1 recruits for ubiquitination are negative regulators of JAs responses.

Three research groups using independent approaches simultaneously identified that a ZIM (Zinc-finger Inflorescence Meristem)-domain family protein had crucial roles in JAs responses in Arabidopsis (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). The physical association between COI1 and a Jasmonate ZIM-domain (JAZ) protein, JAZ1, was detected in a yeast two-hybrid assay and in an *in vitro* pull down assay in the presence of JA-Ile (Thines et al., 2007). The degradation of JAZ3 protein in transgenic plants expressing JAZ3-GFP in wild-type background

was blocked by the proteasome-specific inhibitor MG132, implicating the 26S proteasome in JAs-mediated JAZ3 removal. Moreover, the stabilization of JAZ1-GFP, JAZ3-GFP and JAZ6-GFP in the *coi1* mutant further confirmed that JAZ proteins were direct targets of SCF^{COI1} leading to 26S proteasome-mediated degradation (Chini et al., 2007; Thines et al., 2007). JA-Ile and coronatine, rather than OPDA, JA or MeJA, promoted the COI1-JAZ1 interaction, indicating that the JAZ proteins were the missing link of JAs signaling in Arabidopsis (Thines et al., 2007; Katsir et al., 2008). Different assays used for closely related COI1 and JAZ1 protein interactions in tomato, tobacco and rice established that the mechanism was not unique to Arabidopsis (Thines et al., 2007; Shoji et al., 2008; Sheard et al., 2010; Seo et al., 2011).

Arabidopsis JAZ proteins consisting of 12 members belong to the large family of proteins that share a conserved TIFY sequence within the ZIM motif (Vanholme et al., 2007). This non-DNA-binding TIFY motif mediates homo-and heteromeric interactions between most JAZs (Chung and Howe, 2009). A second defining feature of JAZs is the highly conserved Jas motif near the C terminus which is responsible for protein degradation. The dominant JAs-insensitive mutant *jai3-1* showed impaired transcriptional activation of JAs-responsive genes compared to wild-type Arabidopsis in addition to its phenotypic defect in response to JAs. The *jai3-1* allele encodes a mutant protein JAZ3 lacking a conserved C-terminal Jas domain and is resistant to JAs-triggered degradation (Chini et al., 2007). As for *jai3-1*, high-level expression of *JAZ1Δ3A*, a derivative with a deletion of the conserved Jas motif, blocked JAs responsiveness (Thines et al., 2007; Chung et al., 2008). Overexpression of a natural splice variant of *jasmonate-associated 1* (*JAS1*, identical to *JAZ10*) lacking part of the Jas domain resulted in reduced sensitivity to MeJA and elevated root and shoot growth upon MeJA treatment (Yan et al., 2007). Altogether, the lack of an obvious DNA-binding domain, the nuclear localization, the rapid induction by JAs and the dominant JAs-insensitive phenotype resulting from Jas motif deletion (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Chung et al., 2008; Chung and Howe, 2009) suggest that the hypothetical transcriptional repressor function of JAZs would be indirect by suppressing other DNA-binding transcriptional activators. Furthermore, Arabidopsis null mutants harboring T-DNA insertions in the *JAZ2*, *JAZ5*, *JAZ7* or *JAZ9* genes failed to exhibit JAs-related phenotypes, suggesting that these *JAZ* genes function redundantly (Thines et al., 2007).

The basic helix-loop-helix (bHLH) transcription factor MYC2 (also known as JIN1 or RAP-1) (de Pater et al., 1997; Lorenzo et al., 2004), was responsible for the

activation of a subset of JAs-responsive genes (Lorenzo et al, 2004). MYC2 interacted with most JAZ repressors directly (Chini et al., 2009). Similar to MYC2, the phylogenetically related transcription factors MYC3 and MYC4 interacted *in vivo* and *in vitro* with JAZ repressors (Fernández-Calvo et al., 2011; Niu et al., 2011; Goossens et al., 2015). Current models of JAs signaling indicate that, in the presence of low levels of JAs, the transcriptional activity of MYCs is repressed by JAZ proteins which recruit TOPLESS (TPL) to form a repressor complex either directly (Shyu et al., 2012) or through the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) (Pauwels et al., 2010). Elevated levels of JAs caused by external stimuli promote interaction of JAZ proteins with COI1 and subsequent degradation of JAZs by the 26S proteasome, leading to the release of MYC transcription factors and the activation of primary response genes. Interestingly, in addition to transcriptional activators, JAZ proteins also target transcriptional repressors, such as JAM1, JAM2 and JAM3, to attenuate their transcriptional function (Song et al., 2013; Fonseca et al., 2014; Sasaki-Sekimoto et al., 2014). The balance between repression and activation would lead to an appropriate expression output of JAs-responsive genes, resulting in an appropriate level of JAs responses (Song et al., 2013).

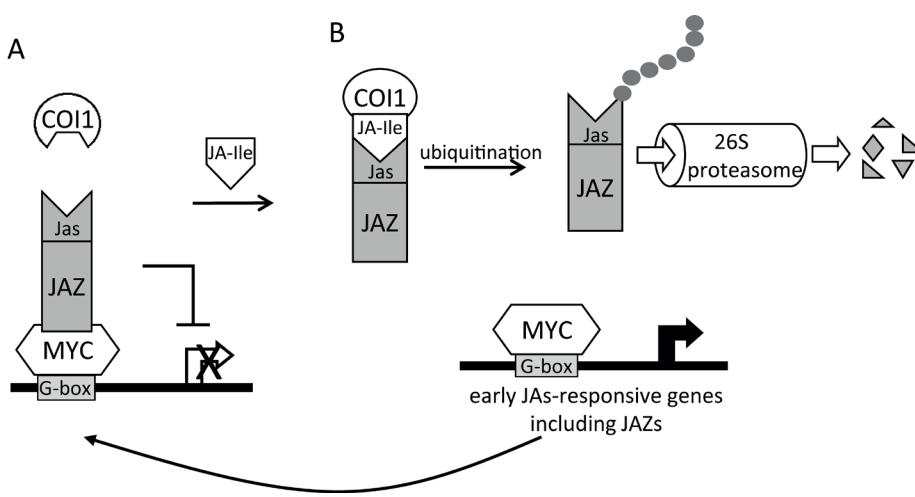

Figure 2. Model for the regulation of JAs-responsive gene expression by MYC and JAZ proteins. (**A**) In the absence of JA-Ile, JAZ proteins interact with MYCs maintaining these transcription factors inactive. (**B**) JA-Ile promotes the interaction between JAZs and COI1, leading to degradation of JAZs by the 26S proteasome presumably via ubiquitination. Subsequently, MYCs are liberated to activate their target genes, including *JAZ* genes, resulting in a negative feedback regulation. See text for other abbreviations.

Transcription factors and JAs responses

I) bHLH-domain Transcription factors

Transcription factors that depend on JAs signaling for activity are the key components for JAs signal transduction processes by regulating expression of downstream genes via specific binding to *cis*-acting elements in the promoters of target genes. The bHLH proteins form a superfamily in Arabidopsis with 162 bHLH-encoding genes (Bailey et al., 2003). The best characterized and most multifunctional bHLH-domain transcription factor involved in JAs signaling pathway is MYC2, which regulates a subset of JAs-responsive genes. The JAs-responsive *MYC2* gene was identified in *jin1* (*jasmonate-insensitive 1*) mutants which originated from a screening for mutants affected in JAs-induced root growth inhibition (Lorenzo et al., 2004). The *jin1/myc2* mutant showed reduced root growth inhibition in response to JAs and constitutive expression of *MYC2* conferred hypersensitivity to JAs. MYC2 displays the general characteristics of the bHLH protein family (Toledo-Ortiz et al., 2003). The bHLH domain of MYC2 is responsible for DNA-binding and the formation of homo- and/or heterodimers with other transcription factors. The G-box (CACGTG) and G-box-related hexamers are the binding sequences of MYC2 in its target promoters (de Pater et al., 1997; Dombrecht et al., 2007 ; Chini et al., 2007). In its amino-terminus, MYC2 contains a putative transcription activation domain (TAD) recruiting the Mediator complex required for transcription initiation and a JAZ interaction domain (JID) for interaction with JAZ proteins (Kazan and Manners, 2013).

In Arabidopsis, MYC2 differentially modulates JAs-dependent responses through direct regulation of its target genes and physical interaction with other transcription factors. As a positive regulator in response to wounding, MYC2 activates the expression of *LOX2* and *TAT1* genes by directly binding to their promoters (Hou et al., 2010). MYC2 positively regulated JAs-mediated resistance to insect pests, such as *Helicoverpa armigera*, and flavonoid biosynthesis possibly via modulating the expression of positive transcriptional regulators in Arabidopsis (Dombrecht et al., 2007). In addition, most *JAZ* genes were constitutively expressed in plants overexpressing *MYC2* and JAs-responsive expression of most *JAZ* genes was reduced in a loss-of-function *myc2* mutant, indicating that MYC2 fine-tunes the JAs signaling pathway by regulating the expression of JAZ repressors (Chini et al., 2007). Further studies have demonstrated that MYC2 directly targeted the G-boxes in the promoters of at least three *JAZ* genes, *JAZ1* (Pauwels and Goossens, 2008), *JAZ2*

(Niu et al., 2011) and *JAZ3* (Chini et al., 2007). In contrast, the genes involved in defenses against pathogens are repressed by MYC2 consistent with the increased resistance to necrotrophic pathogens of the *jin1/myc2* mutant (Lorenzo et al., 2004). The negative effect of MYC2 is due to the MYC2-mediated direct suppression of two genes, encoding ETHYLENE RESPONSE FACTOR 1 (ERF1) and AP2/ERF-domain protein ORA59 (Dombrecht et al., 2007; Zhai et al., 2013), which regulate defense against pathogens. More recently, it has been reported that MYC2 physically interacted with ETHYLENE INSENSITIVE3 (EIN3) to inhibit its DNA binding activity and simultaneously induced the expression of the gene encoding the F-box protein *EIN3 BINDING F-BOX PROTEIN1* (*EBF1*) to promote EIN3 degradation during apical hook development (Song et al., 2014; Zhang et al., 2014).

The two Arabidopsis bHLH-domain proteins MYC3 and MYC4 share high sequence similarity with MYC2, suggesting they probably have similar biological function. Indeed, MYC3 and MYC4 interact *in vitro* and *in vivo* with JAZ repressors and also form homo-dimers or hetero-dimers with each other or MYC2. They are nuclear proteins and have similar DNA binding specificity as MYC2. Analysis of single, double and triple mutants revealed that MYC2, MYC3 and MYC4 regulate both overlapping and distinct functions (Fernández-Calvo et al., 2011; Niu et al., 2011). Moreover, some other bHLH transcription factors including TT8, GL3 and EGL3, essential components of WD-repeat/bHLH/MYB transcriptional complexes mediating diverse plant responses, such as anthocyanin biosynthesis and trichome initiation, are targets of JAZ repressors (Qi et al., 2011). More recently, the bHLH transcription factors JAM1, JAM2 and JAM3 were found to act as transcriptional repressors and to negatively regulate JAs responses by forming protein-protein interactions with JAZs (Sasaki-Sekimoto et al., 2013; Fonseca et al., 2014). The mechanism for the negative regulation of JAs signaling by JAMs may also be based on the competitive binding to the target sequences of MYC2 (Nakata et al., 2013; Song et al., 2013).

II) AP2/ERF-domain transcription factors

The AP2/ERF superfamily is defined by the AP2/ERF domain, which consists of approximately 60 amino acids and is involved in recognizing a GCC (AGCCGCC) motif for DNA binding (Mizoi et al., 2012). In *Catharanthus roseus*, Octadecanoid-derivative Responsive *Catharanthus* AP2-domain (ORCA) proteins, ORCA2 and ORCA3, belong to the AP2/ERF-domain family and the expression of the encoding genes was rapidly induced by MeJA (Menke et al., 1999; van der Fits and Memelink, 2001).

ORCA2 and ORCA3 trans-activated the promoter of the monoterpenoid indole alkaloid (MIA) biosynthesis gene *Strictosidine synthase* (*STR*) through binding to a JAs- and elicitor-responsive element (JERE) containing a GCC motif (Menke et al., 1999; van der Fits and Memelink, 2001). Overexpression of *ORCA3* in transgenic *C. roseus* suspension cells resulted in enhanced expression of several genes encoding enzymes involved in primary and secondary metabolism, including *STR* and, consequently, in increased accumulation of MIA (van der Fits and Memelink, 2000). This demonstrates that the JAs-induced expression of *STR* gene is controlled by the JAs-responsive AP2/ERF-domain transcription factors ORCA2 and ORCA3, providing the first evidence for a link between members of the AP2/ERF-domain family and JAs signaling.

In Arabidopsis, the AP2/ERF transcription factor family comprises 145 members (Sakuma et al., 2002). Based on the observation that *ORCA* genes were induced by JAs, 14 *Octadecanoid-Responsive Arabidopsis AP2/ERF-domain* (*ORA*) genes were identified as being rapidly induced by JAs in a COI1-dependent manner (Atallah, 2005). The expression of *ORA59* was induced by JAs or ethylene (ET) and was synergistically induced by both hormones (Pré et al., 2008). Genome-wide microarray analysis showed that overexpression of *ORA59* activated the expression of several JA- and ET-responsive defense genes, including the defense gene *PLANT DEFENSIN 1.2* (*PDF1.2*). Plants overexpressing *ORA59* were more resistant to infection by the necrotrophic fungus *Botrytis cinerea*. The expression of the AP2/ERF-domain transcription factor *ERF1*, a close paralogue of *ORA59*, was similar to *ORA59* and dependent on JAs and/or ET signals (Lorenzo et al., 2003). Constitutive overexpression of *ERF1* rescued the defense response defect of *coi1* and *ein2* mutants and induced the expression of several genes responsive to both ET and JAs, including *PDF1.2* and *basic chitinase* (*ChiB*) (Lorenzo et al., 2003). In terms of these similarities in gene expression patterns and in target gene sets, as well as their phylogenetic relationship in the AP2/ERF-domain family, ORA59 and ERF1 hypothetically have redundant functions in JAs and ET signal transduction. However, the essential role of ORA59 as an integrator of JAs and ET signals to regulate defense genes was demonstrated with *ORA59*-silenced plants generated by the RNAi approach (Pré et al., 2008). In response to JAs and/or ET, or after infection with the necrotrophic fungi *B. cinerea* or *Alternaria brassicicola*, the expression of defense-related genes such as *PDF1.2*, *hevein-like* (*HEL*) and *ChiB* was blocked in *ORA59*-silenced plants. As expected from the dramatic reduction of defense gene expression, the silenced plants exhibited

more susceptibility to *B. cinerea* infection, further supporting the crucial role of ORA59 in the integration of JAs and ET signals. The activity of ORA59 in defense against necrotrophic pathogens was repressed by JAZ1 via the ZFAR1 and ZFAR2 adaptor proteins (Körbes, 2010; Zhou, 2014).

The AP2/ERF-domain transcription factor ORA47 functions in the regulation of the JAs biosynthesis pathway (Pré, 2006; Chen et al., 2016). Overexpression of the *ORA47* gene conferred obvious JAs-related phenotypes, such as inhibition of growth and anthocyanin production, and induced expression of most JAs biosynthesis genes. JAs measurements in plants overexpressing *ORA47* showed an increase in the amounts of the JA precursor OPDA, JA, the bioactive JA-Ile and 12-hydroxy-JA (Khurshid, 2012). Probably, as a consequence of JAs accumulation, several JAs-responsive defense genes including *VSP1* were upregulated in *ORA47*-overexpressing plants. ORA47 activated the expression of the *AOC2* gene, encoding an enzyme of JAs biosynthesis, via specifically binding to a GCC-like motif in the *AOC2* promoter (Zarei, 2007; Chen et al., 2016). These findings indicate that ORA47 act as an important regulator in the positive JAs-responsive feedback loop by controlling the expression of JAs biosynthesis genes.

AtERF3 and AtERF4 act as repressors and downregulated not only the transcription levels of their target genes but also interfered with the activity of other activating transcription factors (Fujimoto et al., 2000). The expression of *AtERF4* gene was induced by JAs, ET, ABA, wounding or infection with *Fusarium oxysporum* (Fujimoto et al., 2000; McGrath et al., 2005; Yang et al., 2005; Pré, 2006). In untreated *AtERF4*-overexpressing plants, the basic transcript levels of defense genes *PDF1.2* and *ChiB* remained at a similar level as in wild-type plants. A dramatic increase in the *PDF1.2* basal transcript level was found in *erf4-1* mutant plants compared to wild-type plants, whereas *ChiB* transcript level remained unchanged (McGrath et al., 2005). Nevertheless, in response to JAs or ethylene treatment, expression of a subset of defense-related genes, including *PDF1*, was significantly repressed and enhanced in *AtERF4* overexpressing plants and *AtERF4*-silenced plants, respectively (McGrath et al., 2005; Pré, 2006). It is therefore suggested that AtERF4 is able to repress the expression of the same genes that are positively regulated by ORA59 and ERF1 in response to JAs and/or ET. AtERF3 and AtERF4 differ from the JAs-responsive AP2/ERF transcriptional activators by the presence of an ERF-associated amphiphilic repression (EAR) motif in the C-terminal part of the proteins. This motif has been shown confer the repression ability to AtERF3 and AtERF4 because muta-

tion within this motif eliminated the capacity for repression (Ohta et al., 2001). The co-repressor TOPLESS (TPL) was shown to interact directly with the EAR motifs of NINJA and JAZ8 proteins to repress the transcriptional activation of JAs-responsive genes (Pauwels et al., 2010; Shyu et al., 2012). Thus, AtERF3 and AtERF4 probably repress the expression of JAs-responsive genes through both directly binding to the GCC-box in the promoters and the association with the co-repressor TPL.

III) R2R3-MYB transcription factors

MYB transcription factors are widely distributed in plants, and can be classified into four groups, 1R, 2R, 3R and 4R, based on the number of adjacent repeats in the DNA-binding domain. The DNA binding domain consists of one to four imperfect repeats (R1, R2, R3 and R4), where each repeat is about 50-53 amino acids long and consists of three α-helices, with the second and third helices forming a helix-turn-helix structure which can bind the major groove of DNA. The Arabidopsis R2R3-MYB transcription factors MYB21 and MYB24 were identified as key regulators of stamen and pollen maturation processes triggered by JAs (Mandaokar et al., 2006). The *myb21-1* knockout mutant exhibited shorter anther filaments, delayed anther dehiscence and strongly reduced male fertility, which could not be rescued by exogenous JAs. Although the *myb24* mutant was phenotypically wild-type, the *myb21myb24* double mutant exacerbated all three phenotypic aberrations of the *myb21* mutant. The expression of the *MYB21* and *MYB24* genes was induced by JAs (Mandaokar et al., 2006). MYB108, also encoded by a JAs-responsive gene, shared overlapping functions with MYB24 and acted downstream of MYB21 in a transcriptional cascade that mediates stamen and pollen maturation in response to JAs (Mandaokar, 2009). The direct interaction between a select set of JAZ repressors (JAZ1, JAZ8 and JAZ11) and MYB21 and MYB24 revealed a mechanism in which JAs triggers COI1-dependent JAZ degradation to control MYB21 and MYB24 in stamen development (Song et al., 2011). Overexpression of the R2R3-MYB transcription factor *MYB75* restored anthocyanin accumulation and trichome initiation in the *coi1* mutant. JAZ proteins directly interacted with the MYB factors (MYB75 and GL1) of WD-repeat/bHLH/MYB complexes, attenuated their transcriptional function, and subsequently repressed anthocyanin biosynthesis and trichome initiation (Qi et al., 2011).

JAs signal interaction with SA, GAs and Ethylene

Upon changing environmental conditions, JAs and SA are recognized as major defense hormones of plant immunity. However, the hormones ET, GAs, auxin and ABA function as modulators of the plant immune signaling network as well (Pieterse et al., 2012). This so-called hormone crosstalk enables plants to rapidly adapt to external attack and to utilize their limited resources in a cost-efficient manner (Walters and Heil, 2007).

JAs-SA crosstalk

Generally speaking, SA functions as a plant hormone required for innate immunity against biotrophic pathogens, while JAs are effective in the activation of defense against necrotrophic pathogens (Glazebrook, 2005). When plants are infected by biotrophic pathogens, the accumulation of high levels of SA triggers the localization of NON-EXPRESSOR OF PR GENE1 (NPR1) from the cytoplasm to the nucleus. In the nucleus, NPR1 binds to TGA (TGACG motif binding) transcription factors, initiating the expression of a set of defense-related genes including the *PR* (*Pathogenesis-Related*) genes. Many reports described the antagonistic interplay between SA and JAs. Arabidopsis unable to accumulate SA showed enhanced expression of JAs-responsive genes *PDF1.2*, *LOX2* and *VSP* in response to infection by *Pseudomonas syringae* pv *tomato* DC3000. Analysis of the Arabidopsis mutant *npr1*, which was impaired in SA signal transduction, revealed that the regulatory protein NPR1 was required in the SA-mediated suppression of JAs-regulated genes (Spoel et al., 2003). SA suppressed JAs signaling downstream of the action of the SCF^{COI1}-JAZ complex by targeting JAs-responsive GCC promoter motifs through the reduction of accumulation of ORA59 protein (van der Does et al., 2013). Treatment with exogenous JAs inhibited the expression of several SA-dependent *PR* genes, suggesting that JAs also suppressed SA signaling (Niki et al., 1998). Arabidopsis *coi1* and *mpk4* mutants, which were impaired in JAs-responsive gene expression, exhibited increased SA levels, constitutive expression of *PR1* and enhanced resistance against *P. syringae* (Petersen et al., 2000; Kloek et al., 2001). However, treatment of Arabidopsis with low concentrations of JAs and SA resulted in a synergistic effect on the JAs- and SA-responsive genes *PDF1.2* and *PR1*, respectively, implying that the outcome of JAs-SA interaction is dose-dependent (Mur et al., 2006). The transcription factor WRKY70 acts as a node of convergence for integration of JAs-SA signaling. Expression of *WRKY70* was activated by SA but suppressed by JAs. Overexpression of *WRKY70* in Arabidopsis up-regulated *PR* genes and down-regulated *PDF1.2* gene, leading to

enhanced resistance to biotrophic pathogens and enhanced susceptibility to necrotrophic pathogens (Li et al., 2004). Recently, the JAs-induced R2R3-MYB transcription factor AtMYB44 was reported to modulate the antagonistic JAs-SA interaction via directly activating expression of *WRKY70* through a conserved sequence in the *WRKY70* promoter (Shim et al., 2013).

JAs-GAs crosstalk

GAs play essential roles in controlling plant growth and development by regulating the degradation of growth-repressing DELLA proteins (Sun, 2010). Like JAs signaling, binding of GAs to the receptor GA INSENSITIVE DWARF1 (GID1) promotes the $SCF^{SLY1/GID1}$-DELLA interaction, presumably resulting in ubiquitination and leading to degradation of DELLA repressors and the subsequent de-repression of different transcription factors, such as the positive regulators PHYTOCHROME-INTERACTING FACTORs (PIFs) involved in GAs-mediated plant growth promotion. The quadruple-DELLA mutant was partially insensitive to gene induction by JAs and, moreover, the GAs-deficient mutant *ga1* showed upregulated expression of JAs-responsive genes, suggestive of a positive role of DELLA proteins in JAs signaling (Navarro et al., 2008; Hou et al., 2010). A 'relief of repression' model was proposed for the JAs-GAs interaction, in which DELLAs competed for binding to JAZ1 with the key transcriptional activator of JAs signaling, MYC2, and, thus, liberated MYC2 to regulate its target genes (Hou et al., 2010). Consistent with this model, induction of primary JAs-responsive genes, including *LOX2*, *TAT1* and *VSP2*, was enhanced in transgenic plants overexpressing *RGL3*, encoding a DELLA protein, and reduced in the *rgl3-5* mutant (Wild et al., 2012). On the other hand, JAZ9 inhibited the interaction of the DELLA protein RGA with PIF3, indicating that JAs prioritized defense over growth by interfering with the GAs signaling cascade through the COI1-JAZ-DELLA-PIF signaling module (Yang et al., 2012). In addition, DELLAs positively regulated JAs/ET-mediated resistance to the necrotrophic fungus *B. cinerea* via preventing inhibitory JAZ1-ZFAR interaction with ORA59 and enhancing the activity of ORA59 on its target *PDF1.2* promoter (Zhou, 2014). Interestingly, JAs and GAs can also act synergistically to promote stamen and trichome development. GAs were found to promote JAs biosynthesis through DELLAs to control the expression of *MYB21*, *MYB24*, and *MYB57*, which in turn promoted stamen filament growth (Cheng et al., 2009). GAs and JAs induced degradation of DELLAs and JAZ proteins to coordinately activate the WD-repeat/bHLH/MYB complex and synergistically and mutually dependently

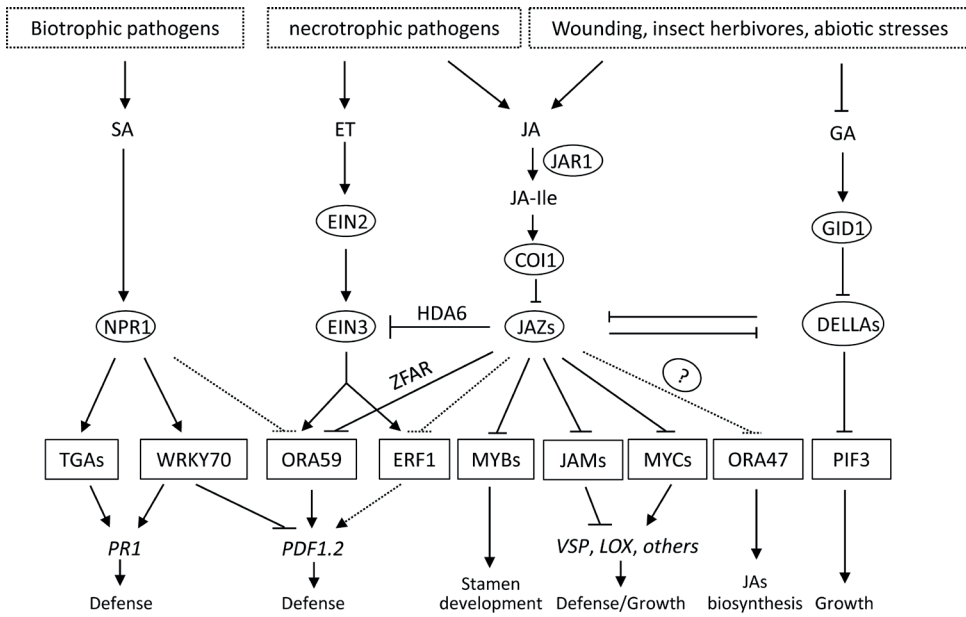

Figure 3. A model for the roles of transcription factors in the stress-responsive network involving JAs, ET, GAs and SA signaling. Attack by wounding, insects and necrotrophic pathogens induces the synthesis of JA, which can be converted to the biologically active JA-Ile by JAR1. Perception of JA-Ile by its receptor COI1 triggers the degradation of JAZ repressors, leading to the release of downstream transcription factors, such as ORA59, ORA47, MYB21/24, MYC2/3/4 and JAM1/2/3, and the regulation of JAs-responsive genes. R2R3-MYB transcription factors MYB21 and MYB24 are positive regulators of stamen development. The bHLH-type transcription factors MYC2/3/4 and JAM1/2/3 antagonistically regulate various JAs-related responses. The AP2/ERF-domain transcription factor ORA47 functions in the regulation of JAs biosynthesis and is hypothesized to be repressed by JAZ family members via an adaptor protein (indicated by the dashed lines). Infection with necrotrophic pathogens simultaneously induces ET together with JAs, resulting in the synergistic induction of *ORA59* and *ERF1* genes depending on EIN3 and COI1. ORA59 repressed by the JAZ1-ZFAR complex is the key regulator of JAs/ET-responsive defense gene *PDF1.2*, whereas the role of ERF1 in gene regulation remains unclear and awaits analysis of a knockout mutant (indicated by the dashed lines). JAZ repressors interact with EIN3 and repress the transcriptional activity of EIN3 via recruiting HDA6. GAs regulate plant growth and development through the degradation of DELLA repressors, which is mediated by the $SCF^{SLY1/GID1}$ complex and the 26S proteasome. The interaction between JAZ and DELLA proteins mediates JAs-GAs crosstalk, providing the molecular switch for the balance between plant growth and defense. SA signaling is generally triggered by biotrophic pathogens, in which nuclear NPR1 binds TGA transcription factors to initiate SA-associated responses. SA suppresses JAs signaling by targeting JAs-responsive GCC promoter motifs through the reduction of accumulation of ORA59 protein. On the other hand, SA induces the expression of *WRKY70* in an NPR1-dependent manner to repress the expression of *PDF1.2*.

induced trichome initiation (Qi et al., 2014).

JAs-ET crosstalk

ET is another important plant hormone in many aspects of the plant life cycle and in response to environmental stimuli. ET is perceived by a family of five membrane-localized receptors in Arabidopsis, which activate the downstream negative regulator Raf-like kinase CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1) through physical interaction in the absence of ET. Binding of ET inactivates the receptors, resulting in inactivation of CTR1, which allows EIN2 to positively regulate the stability of the transcription factors EIN3, EIN3-like 1 (EIL1) and EIL2 in the nucleus to activate ET-responsive genes (Wang et al., 2002). An Arabidopsis microarray experiment showed that nearly half of the ET induced genes were also induced by JAs treatment, indicating that JAs and ET coordinately regulated many defense-related genes (Schenk et al., 2000). As mentioned above, JAs and ET synergistically induced the expression of *ORA59*, *ERF1* and *PDF1.2* in response to necrotrophic pathogens. Nevertheless, in a JAs or ET insensitive mutant (*coi1* or *ein2*, respectively), JAs and ET alone or in combination failed to induce the expression of these defense genes (Lorenzo et al., 2003; Pré et al., 2008). Crosstalk between JAs and ET was reported to be mediated through the direct interaction of JAZ proteins (JAZ1, JAZ3 and JAZ9) with two positive transcription factors for ET responses, EIN3 and EIL1, inhibiting the activity of these regulators by recruitment of the co-repressor HDA6 (HISTONE DEACETYLASE6) (Zhu et al., 2011). Therefore, ET-mediated EIN3/EIL1 stabilization and JAs-mediated EIN3/EIL1 release from JAZ repression resulted in the synergistic activation of *ERF1*, *ORA59* and their downstream target genes such as *PDF1.2*, providing a plausible explanation for the synergy in many JAs/ET-regulated responses (Zhu et al., 2011).

Conclusions

Knowledge about the biosynthesis, signaling and regulation mechanisms of JAs has substantially increased in the last two decades. JAs biosynthesis showed self-activation, in which the products positively regulate the expression of genes encoding enzymes involved in JAs biosynthesis. Currently, the JA conjugate JA-Ile has been exclusively identified to act as the bioactive ligand to promote JAs signaling by enhancing the interaction of JAZ with the SCF^{COI1} complex. In the presence of low levels of JAs, JAZ proteins repress the expression of JAs-responsive genes via interaction with transcription activators such as MYC2, MYC3 and MYC4. In response to envi-

ronmental stimuli, the bioactive JA-Ile is quickly synthesized and perceived by its receptor COI1 to form the SCF^{COI1}-JA-Ile-JAZ complex, resulting presumably in the ubiquitination and in the degradation of JAZ repressors and the de-repression of primary response genes. JAs do not exert the function independently but cooperatively with SA, GAs and ET signals. The positive and negative regulatory components of hormone pathways are potential targets to modify hormone crosstalk during disease and defense. Several important mediators of hormone crosstalk are transcription factors or repressors, implying that crosstalk is predominantly executed in the nucleus downstream of signal transduction, at the level of gene transcription.

Outline of this thesis

JAs are crucial plant signaling molecules that regulate defense responses against wounding, insects and necrotrophic pathogens. Changing environmental conditions triggers quick biosynthesis, perception and transduction of JAs signals. Several transcription factors have been identified that appear to be involved in the expression of JAs-responsive genes, including ORA59, ERF1, ORA47 and MYCs. Transcriptional regulation of JAs biosynthesis in a positive feedback loop has been repeatedly described, however the regulatory mechanism behind this positive feedback loop remains to be elucidated. The studies described in this thesis focused on the functional analysis of the bHLH-domain transcription factors MYC2, MYC3 and MYC4 in the auto-regulatory loop in JAs biosynthesis in Arabidopsis.

Chapter 2 describes the roles of MYC proteins in the regulation of JAs biosynthesis genes. Induction of a large number of genes involved in JAs biosynthesis in response to wounding or MeJA treatment was obviously attenuated in the *myc234* triple mutant compared to wild-type Arabidopsis. MYC proteins bound to a G-box (CACGTG) sequence in the *AOC2* promoter *in vitro* and activated the expression of *AOC2* through direct binding to this G-box sequence *in vivo*. In addition, MYCs and ORA47 additively controlled the activity of promoters of the JAs biosynthesis genes *LOX2*, *AOS*, *AOC2* and *OPR3*. These results indicated that MYCs act as key positive regulators of the auto-stimulatory loop in JAs biosynthesis.

Chapter 3 describes the roles of MYC proteins in the JAs-responsive expression of *ORA47*. The results showed that MYC proteins interacted with a single G-box in the *ORA47* promoter *in vitro*. Transient assays revealed that this G-box sequence was essential for MYC-mediated activation of the *ORA47* promoter and another G-box and a G-box-like sequence contributed to a higher expression level

of the *ORA47* promoter. Triple knockout of the *MYC* genes or overexpression of a stable JAZ1 derivative abolished JAs-responsive *ORA47* expression, demonstrating the crucial role of the MYC-JAZ module in the regulation of *ORA47* expression.

Chapter 4 describes the effect of DELLA proteins on the regulation of JAs biosynthesis genes in Arabidopsis. All five DELLAs have been reported to interact with MYC2 directly and to interfere with the MYC2-mediated JAs signaling output. Via yeast two-hybrid screening two members of the Arabidopsis DELLA protein family, RGA and GAI, were identified as ORA47 interactors. RGA and GAI slightly promoted the activity of ORA47 in the activation of the *AOC2* promoter, which was partially attenuated by co-expression of JAZ1. RGA and GAI significantly enhanced the activation of the *ORA47* promoter through interaction with MYC2. The expression of *LOX2*, *OPR3* and *AOC2* was not affected in the quintuple *della* mutant or in plants constitutively overexpressing RGA or GAI.

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