Functional analysis of ORA47, a key regulator of jasmonate biosynthesis in arabidopsis

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SUMMARY

Jasmonic acid (JA) and related oxylipins, collectively known as jasmonates (JAs), are key regulators of plant development and plant responses to abiotic and biotic challenges. The major bioactive JAs is the amino acid conjugate jasmonoyl-isoleucine (JA-Ile). JA-Ile is perceived by the F-box protein CORONATINE-INSENSITIVE1 (COI1) which is part of an SCF complex with putative E3 ubiquitin ligase activity. This leads to degradation of JAZ repressor proteins, which sets in motion gene expression programmes.

Genes involved in defense of Arabidopsis plants against wounding and insect herbivory are controlled by the transcription factors MYC2, MYC3 and MYC4. JAZ can bind to MYC and are thought to repress their activity. JAZ degradation is thought to release these transcription factors from repression leading to expression of defense genes.

The majority of the enzymes acting in the octadecanoid pathway for biosynthesis of JAs have been identified and the corresponding genes are known. The expression of all these biosynthesis genes including *LOX* and *AOC* is induced by treatment with exogenous JA or methyl-JA (MeJA), indicating that JAs signaling is amplified by a positive feedback loop initiated by JAs. How this feedback loop is controlled at the transcriptional level is not well understood. Candidate transcription factors were reported in the literature. In a transient assay to screen for activators MYC2 and the AP2/ERF-domain transcription factor ORA47 were able to activate the *LOX3* promoter in tobacco protoplasts. Whereas MYC2 has been extensively studied and is one of the major JAs-responsive transcription factors, little is known about the function of ORA47. The expression of the *ORA47* gene was reported to be induced by JA in a COI1-dependent manner.

The studies described in this thesis were focused on the functional analysis of ORA47 in Arabidopsis. The aim of the research was (1) to investigate whether ORA47 regulates the positive feedback loop in JAs biosynthesis, (2) to determine its target genes, (3) to establish how ORA47 is regulated at the protein level, and (4) to understand the regulation of JAs-responsive *ORA47* gene expression.

The current knowledge of the octadecanoid pathway for biosynthesis of JAs and of the different components of the JAs signaling pathway are reviewed in **Chapter 1**.

Chapter 2 describes the role of ORA47 in JAs biosynthesis. Overexpression of the *ORA47* gene conferred JAs-related phenotypes, such as inhibition of growth and anthocyanin production, and induced the expression of all JAs biosynthesis genes tested. JAs measurements in *ORA47*-overexpressing plants showed an increase in the amounts of the JA precursor 12-oxophytodienoic acid (OPDA), JA, the bioactive JA-Ile and the inactive derivative 12-hydroxy-JA. Probably as a consequence of JAs production several JAs-responsive defense genes were upregulated in *ORA47*-overexpressing plants**.** The results indicate that ORA47 acts as the key regulator in the positive feedback loop by controlling the expression of the JAs biosynthesis genes.

Chapter 3 describes the identification of candidate target genes of ORA47. To distinguish between direct target genes and secondary genes responding to JAs an inducible *ORA47* overexpression system was used in the wildtype background and in *aos* mutant plants, which are unable to synthesize JAs. Changes in transcript abundance of 24,000 genes in response to switching on *ORA47* expression in the two genetic backgrounds were determined using Arabidopsis whole genome Affymetrix (ATH1) gene chips. Unexpectedly, most JAs biosynthesis genes responded to ORA47 overexpression to a much lesser degree in the mutant background. The JAs biosynthesis gene *JASMONATE RESISTANT 1* (*JAR1*) responded equally strong in the wildtype and mutant background, making it a strong candidate for an ORA47 target gene. It is hypothesized that the other JAs biosynthesis genes are also direct target genes of ORA47, but that they are subject to a second layer of JAs-responsive regulation.

Chapter 4 describes the characterization of proteins that interact with ORA47 and may regulate its activity. The ORA47 target gene *AOC2* is a primary JAs-responsive gene, indicating that the activity of ORA47 is regulated by a repressor protein that is degraded in a JAs-responsive and COI1-dependent manner. Via yeast two-hybrid screening and pull-down assays all 5 members of

the Arabidopsis BTB-TAZ protein family except BT2 were identified as ORA47 interactors. ORA47 did not directly interact with JAZ proteins, but all BT proteins except BT2 did, which is compatible with a scenario where BT proteins act as adaptors between ORA47 and JAZ. BT4 and BT5 partially repressed ORA47 activity in a transient trans-activation assay, whereas JAZ1 had no effect. Analysis of quadruple *bt* knockout plants having either a wildtype *BT2* or *BT3* gene showed no effect on basal or JA-responsive expression of *AOC2*. From these results it was concluded that BT proteins are not the hypothetical repressors or adaptor proteins that were the target of the research described in this chapter.

Chapter 5 describes the identification of the promoter element(s) and the transcription factor(s) responsible for JAs-responsive expression of the *ORA47* gene. Based on literature data the hypothesis that *ORA47* is regulated by the functionally redundant JAs-responsive transcription factors MYC2, MYC3 and MYC4 was explored. The results show that the MYC proteins can bind to a single G-box in the *ORA47* promoter. Triple knockout of the *MYC* genes or overexpression of a stable JAZ1 derivative abolished JA-responsive *ORA47* expression, demonstrating the crucial role of the MYC-JAZ module in the regulation of *ORA47* expression.

The aim of the studies described in this thesis was to determine the role of the AP2/ERF-domain transcription factor ORA47 in the transcriptional regulation of the JAs-responsive positive feedback loop in JAs biosynthesis.

In short, the following new results were obtained. Overexpression of ORA47 resulted in increased expression of all JAs biosynthesis genes tested and in elevated levels of several JAs including JA and JA-Ile. The JAs biosynthesis genes are probably direct target genes of ORA47. ORA47 does not directly interact with JAZ repressors in yeast two-hybrid assays. The JA-responsive expression of the *ORA47* gene is controlled by the transcription factors MYC2, MYC3 and MYC4 which interact with a G-box in the ORA47 promoter.

One important aim of the thesis research was to isolate the hypothetic repressor protein which is thought to control ORA47 activity in a manner

similar to the MYC-JAZ interaction. Unfortunately the research failed to identify this repressor. Assuming that JAZ are the only proteins which are degraded via SCF^{COI1} activity, the preferred hypothesis is that ORA47 is regulated via interaction of its C-terminal domain with an adaptor protein that recruits certain members of the JAZ repressor family.