ORA EST : functional analysis of jasmonate-responsive AP2/ERF-domain transcription factors in Arabidopsis thaliana

Pré, M.

Chapter 6

Summary and general discussion

Plants respond to environmental stress or pathogen attack by producing a number of endogenous secondary signaling molecules including jasmonic acid (JA), ethylene and salicylic acid. The biosynthesis of one or a combination of these hormones and subsequent transduction of the signals triggers dramatic modifications in transcription leading to adapted defense responses. In response to certain pathogens, JA and ethylene cooperate to synergistically induce the expression of a large number of defense genes and such a concerted action of JA and ethylene is though to be responsible for the establishment of a fine-tuned complex defense response.

Jasmonic acid and related compounds, collectively referred to as jasmonates, form a family of cyclopentanone derivatives synthesized from linolenic acid via the octadecanoid pathway (Turner et al., 2002; Atallah and Memelink, 2004). These molecules regulate several aspects of plant growth and development as well as responses to many biotic and abiotic stresses (Turner et al., 2002). In recent years, several lines of evidence have shown that several members of the jasmonate family, including the JA-precursor OPDA, are biologically active and can have overlapping as well as distinct roles as signal molecules.

At present, it is largely unknown what are the molecular players regulating JA production in response to stress signals. In contrast, the mechanisms whereby JA signaling triggers gene expression are becoming well documented. In *Catharanthus roseus*, JA induces the expression of two genes encoding transcription factors called Octadecanoid-Responsive Catharanthus AP2/ERF-domain (ORCA) proteins (Menke et al., 1999; van der Fits and Memelink, 2000 and 2001), which belong to the AP2/ERF-domain protein family. The ORCAs mediate the JA response by positively regulating the expression of several JA-responsive genes via direct binding to a JA-responsive element in the promoter of these genes (Menke et al., 1999; van der Fits and Memelink, 2001). Based on these observations, it was postulated that JA-responsive expression of a subset of genes in the model plant *Arabidopsis thaliana* is also mediated by specific members of the AP2/ERF-domain transcription factor family, and that the corresponding genes are also expressed in a JA-responsive manner. In Arabidopsis, the AP2/ERF-domain transcription factor family was reported to comprise 124 proteins. In a family-wide screening, Atallah (2005) previously characterized an initial set of 10 genes, called *Octadecanoid-Responsive Arabidopsis AP2/ERF* (*ORA*) genes, which were rapidly induced by JA treatment in young seedlings, and later on found 4 additional *ORA* genes.

The studies described in this thesis are focused on the functional characterization of the 10 ORA proteins identified initially with emphasis on their role in JA-responsive gene expression and in the JA signaling network. **Chapter 2** describes the role of the transcription

factor ORA59 in the network involving JA and ethylene defense signaling pathways. *ORA59* gene expression was induced by JA or ethylene, and synergistically induced by both hormones (Atallah, 2005). Analysis of the JA-insensitive (*coi1-1*) and ethylene-insensitive (*etr1-1* and *ein2-1*) mutants revealed that expression of the *ORA59* gene in response to JA (Atallah, 2005) or ethylene (this study) required both JA and ethylene signaling pathways simultaneously. Genome-wide microarray analysis showed that overexpression of the *ORA59* gene resulted in increased expression of a large number of JA- and ethylene-responsive genes, including several genes involved in defense or in primary and secondary metabolism. Several defense genes, including *PDF1.2* and *HEL*, are expressed as a result of ORA59 overexpression even in a *coi1-1* mutant background, which is consistent with the hypothesis that ORA59 functions downstream from COI1. Plants overexpressing *ORA59* were more resistant to infection with the necrotrophic fungus *Botrytis cinerea* compared to infected wild-type plants. Plants overexpressing *ERF1*, a gene encoding a related member of the AP2/ERF family, were previously shown to induce *PDF1.2* and *HEL* gene expression (Solano et al., 1998; Lorenzo et al., 2003) and to be more resistant to *B. cinerea* (Berrocal-Lobo et al., 2002). Similar to *ORA59* expression, *ERF1* gene induction by JA and/or ethylene was dependent on both JA and ethylene signaling pathways (Lorenzo et al., 2003). These similarities in gene expression patterns and in putative target genes, as well as the fact that they are the closest mutual homologues in the AP2/ERF-domain family, suggest that ORA59 and ERF1 have redundant functions in JA and ethylene signal transduction. However, the essential role of ORA59 as an integrator of the JA and ethylene signals leading to regulation of defense genes was demonstrated with plants silencing the *ORA59* gene via an RNAi approach. In response to JA and/or ethylene, or after infection with the fungi *B. cinerea* or *Alternaria brassicicola,* expression of *PDF1.2* and other defense genes was blocked in *ORA59*-silenced plants. Induction of the *ERF1* gene in the *ORA59*-silenced plants was identical to that in control plants in response to JA and/or ethylene, showing that RNAi-mediated silencing was specific for *ORA59*. As expected from the dramatic effect on defense gene expression, the silenced plants were also more susceptible to *B. cinerea* infection. The results demonstrate that ORA59 integrates JA and ethylene signal inputs to coordinate the appropriate gene expression response directed against pathogen attack and thereby provide new insight in the nature of the molecular components involved in the crosstalk between these two hormones.

The data presented in Chapter 2 also emphasize the necessity of interpreting results obtained by constitutive overexpression of a gene in conjunction with data from other complementary approaches, such as inducible overexpression, as well as gene silencing by RNAi or insertional knock-out. Constitutive overexpression of *AtERF1* (Atallah, 2005),

AtERF2 (Brown et al., 2003; Atallah, 2005), *ERF1* (Solano et al., 1998) and *ORA59* (this study) in Arabidopsis all resulted in increased *PDF1.2* expression. However, using two different approaches, we found that only ORA59 and ERF1 were able to function as transcriptional activators of *PDF1.2* gene expression, indicating that *PDF1.2* is not a direct target gene of the other AP2/ERF-domain family members, but is likely to be transcriptionally upregulated as a result of general stress due to overexpression. An evaluation of whether ERF1 has essential roles or whether it is an expendable functionally redundant transcription factor awaits analysis of *ERF1* knock-out mutants.

Chapter 3 describes the functional characterization of the transcription factor ORA47. Constitutive overexpression of the *ORA47* gene in Arabidopsis resulted in an extreme dwarf phenotype with production of anthocyanins at the shoot apex. This phenotype was similar to that of wild-type plants grown in the presence of JA. Therefore we speculated that plants overexpressing *ORA47* exhibited constitutive JA responses. To be able to study the function of ORA47, we switched to an inducible overexpression system. Induced overexpression of *ORA47* led to the activation of a large number of genes encoding JA biosynthetic enzymes, including AOC3, AOS and LOX2. Consistent with this finding, induced plants overexpressing *ORA47* exhibited a 2- to 4-fold higher level of the JA-precursor 12-oxo-phytodienoic acid (OPDA) compared to induced control plants. Surprisingly, JA content in *ORA47*-overexpressing plants remained similar to that observed in the control plants. The first steps of JA biosynthesis occur in the chloroplasts and lead to the formation of OPDA (Turner et al., 2002). Subsequently, OPDA needs to be targeted to the peroxisome to undergo further enzymatic modifications to yield JA. Overexpression of *ORA47* led to induced expression of the genes encoding biosynthesis enzymes, including OPR3 and the β-oxidation enzymes, involved in the conversion of OPDA to JA. It is not clear why elevated amounts of OPDA, together with the activation of genes coding for downstream JA biosynthesis enzymes, did not lead to higher levels of JA in the transgenic plants. We speculate that the OPDA produced in *ORA47*-overexpressing plants failed to be converted to JA due to a lack of transport of OPDA from the chloroplasts to the peroxisomes.

In addition to the JA biosynthesis genes, induction of *ORA47* expression led to increased expression of several JA-responsive defense genes, most likely indirectly as a consequence of OPDA production. We speculate that ORA47 controls oxylipin biosynthesis via direct transcriptional regulation of the JA biosynthesis genes, although this remains to be demonstrated. Overexpression of *ORA47* in the *coi1-1* mutant background did not result in activation of the JA biosynthesis genes *AOC3*, *AOS* and *LOX2*. It is possible that the activity of the ORA47 protein requires post-translational modifications or protein-protein interactions that depend on JA signaling via COI1.

The expression of all JA biosynthesis genes, including *LOX2*, *AOS* and *AOC*, was shown to be induced by treatment with exogenous JA or MeJA (Turner et al., 2002). These results indicate the existence of a positive feedback regulatory mechanism for oxylipin biosynthesis. The results described in this chapter indicate that it is likely that ORA47 is involved in the regulation of this auto-stimulatory loop. It is also possible that, ORA47 not only functions in JA-responsive oxylipin biosynthesis within an auto-stimulatory loop, but also in the initial oxylipin biosynthesis in response to the primary stress signal. This however remains to be investigated. In any case the AP2/ERF-domain protein ORA47 is the first transcription factor shown to control the biosynthesis of regulatory oxylipin signals. ORA47 does not appear to have a unique function, since in plants impaired in *ORA47* expression, the JA-induced expression of the JA biosynthesis genes was comparable to the expression in wild-type plants, indicating that (an) additional transcription factor(s) regulate(s) the expression of the JA biosynthesis genes in response to JA.

In **Chapter 4**, the attention was focused on ORA37. The transcription factor ORA37 differs from the other JA-responsive ORAs by the presence of an ERF-associated amphiphilic repression (EAR) motif in the C-terminal part of the protein. The EAR motif has been shown to function as an active repressor of transcription (Otha et al., 2001). The *ORA37* gene, also referred to as *AtERF4*, is induced by JA (Atallah, 2005), ethylene (Fujimoto et al., 2000) or wounding (Cheong et al., 2002). Overexpression of *ORA37* had no effect on the basal transcript level of several JA-responsive genes in untreated plants. However, upon JA and/or ethylene treatment, *ORA37*-overexpressing plants showed significantly lower induction of a subset of JA- and ethylene-responsive genes, including the defense genes *PDF1.2, HEL* and *ChiB,* compared to control plants treated similarly. On the other hand, plants in which *ORA37* expression was silenced via RNAi showed increased *PDF1.2, HEL* and *ChiB* transcript levels after JA- and/or ethylene-treatment compared to control plants, corroborating the complementary results obtained with *ORA37*-overexpressing plants. This demonstrates that ORA37 plays a role in JA and ethylene signaling by repressing the expression of a number of genes in response to JA and/or ethylene. The same genes were shown to be positively regulated by ORA59 (Chapter 2). Overexpression or silencing of the *ORA37* gene had no effect on the expression of *ORA59* in response to JA and/or ethylene, indicating that ORA37 does not regulate *ORA59* expression. We speculate that ORA37 and ORA59 act antagonistically on the regulation of expression of a same subset of JA- and ethylene-responsive genes.

In addition, overexpression of the *ORA37* gene resulted in enhanced JA-induced expression of a distinct subset of JA-responsive genes, including *VSP1* and *CYP79B2*. This indicated that the presence of ORA37 positively regulated the expression of these genes in

response to JA treatment. It is not clear how the positive effect of *ORA37* overexpression on JA signaling for this gene subset is operating at the molecular level, but assuming that ORA37 always acts as a repressor, the positive effect is hypothesized to be caused by the repression of a repressor. The ethylene signaling pathway was shown to repress the wound-induced expression of several wound-responsive genes, including the *VSP1* and *CYP79B2* genes (Rojo et al., 1999; Mikkelsen et al., 2000). Overexpression of the ethylene-responsive *ERF1* gene has been shown to inhibit the expression of the *VSP2* gene in response to JA (Lorenzo et al., 2004). JA-induced expression of the *VSP2* gene is controlled by the bHLHZIP-type transcription factor AtMYC2 (Figure 1; Lorenzo et al., 2004). It was therefore suggested that the negative regulation of the *VSP2* gene by ethylene is executed through ERF1, although the molecular relationships between the activator AtMYC2 and the repressor ERF1 on JA-responsive *VSP2* expression remains to be characterized. It is possible that ORA37 antagonizes the ERF1-mediated negative effect of ethylene on the expression of a subset of JA-responsive genes, including *VSP* genes. ORA37 and AtMYC2 seem to positively regulate the same subset of JA-responsive genes. However, overexpression of *AtMYC2* is sufficient to activate *VSP2* expression (Lorenzo et al., 2004), which is not the case in *ORA37*-overexpressing plants. Unraveling the mechanisms whereby ORA37 operates is now a great challenge.

Therefore, JA and ethylene induce both activators (e.g. ORA59, AtMYC2 and ERF1) and repressors (e.g. ORA37) of gene expression. The functional importance of the simultaneous induction of both positive and negative regulators by JA and ethylene remains unclear. The balance between AP2/ERF-domain activators and repressors on common target promoters may provide a mechanism for switch-like transcriptional control.

Based on the results obtained from research conducted in *Catharanthus roseus* with ORCA transcription factors (Menke et al., 1999; van der Fits and Memelink, 2000; 2001), the working hypotheses underlying the thesis studies was that JA-responsive gene expression in Arabidopsis is also mediated by members of the AP2/ERF-domain transcription factor family, and that the corresponding genes are also induced by JA. In *Arabidopsis thaliana,* 14 *ORA* genes were shown to be induced by JA in young seedlings (Atallah, 2005). This raised the questions whether ORA proteins were indeed involved in the JA signal transduction network and whether ORA proteins were having identical or distinct functions, if any, in JA signaling. Some answers to these questions have been provided in the previous chapters. The results demonstrated that ORA37, ORA47 and ORA59 transcription factors are important molecular players regulating discrete responses in the JA signaling network. In **Chapter 5**, attempts to assign functions to the other ORA transcription factors and to address the question of putative functional redundancy between ORAs are described. The ten *ORA* genes which

were initially identified were individually overexpressed in plants. To be able to identify direct ORA target genes and to avoid non-specific gene activation that may occur as a result of constitutive overexpression, transgenic plants overexpressing the *ORA* genes under the control of an estradiol-inducible promoter (XVE system; Zuo et al., 2000) were constructed. Analyses of transgene expression in the presence or absence of inducer demonstrated that the XVE system was tightly controlled. Moreover, transgene expression in induced XVE plants reached transcript levels that were similar to or even higher than those observed in transgenic plants in which the transgene is controlled by the strong constitutive CaMV 35S promoter.

A number of putative candidate target genes, which are known to be responsive to JA and/or ethylene, were selected and their expression was measured in the different transgenic lines overexpressing individual *ORAs*. These genes encode proteins involved in defense against biotic or abiotic stress, JA biosynthesis or primary and secondary metabolism. Gene expression profiling in the different XVE-*ORA* lines allowed clustering of the putative target genes in four groups. Genes from group I were induced in XVE-*ORA1*, XVE-*ORA33*, XVE-*ORA47* and XVE-*ORA59* transgenic lines. Genes from group II were induced in XVE-*ORA1*, XVE-*ORA33* and XVE-*ORA47* transgenic lines. Genes from group III were exclusively induced in XVE-*ORA47* transgenic lines, whereas genes from group IV were exclusively induced in XVE-*ORA59* transgenic lines. In addition, there was a group of tested genes which were not induced in any of the ORA lines. These results indicated that several ORA proteins can regulate the same set of genes (e.g. genes from group I and II), suggesting a possible functional redundancy between ORA1, ORA33, ORA47 and ORA59. However, up-regulation of the majority of genes in XVE-*ORA47* plants is likely to be caused indirectly as a result of oxylipin production. Therefore, ORA47 is likely to play a role other than those of ORA1, ORA33 and ORA59 in JA signaling.

Despite the possibility of similar functions among ORAs, the presence of multiple groups of genes with differential expression profiles suggests that ORA1, ORA33 and ORA59 regulate distinct sets of JA-responsive genes although some overlap occurs among ORA-regulated genes.

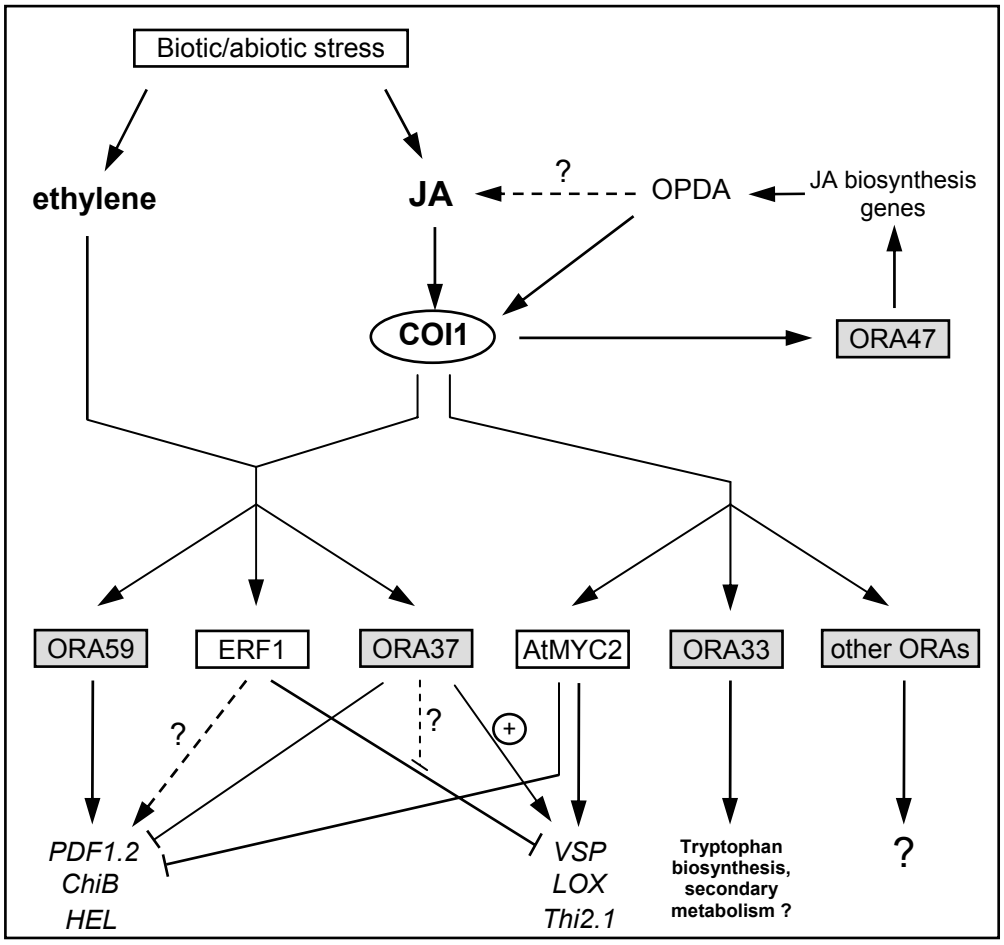

Figure 1. Role of the ORA transcription factors in the stress-responsive network involving the JA and ethylene signaling pathways. Different types of biotic or abiotic stress, such as infection with certain pathogens or wounding, induce the synthesis and subsequent activation of the JA and ethylene signaling pathways. JA (and/or related oxylipins) activates the expression of several transcription factors, including ORAs, via COI1, a central regulator of all JA-dependent responses. The transcription factor ORA47 acts as a regulator of the positive feed-back loop of JA. ORA47 activates JA biosynthesis genes, resulting in production of OPDA, a bioactive precursor of JA. This results in the amplification of the signal initiated by JA. The putative conversion of OPDA to JA is indicated by a question mark. The transcription factor ORA33 activates genes involved in tryptophan biosynthesis and secondary metabolism. The bHLHZIP-type transcription factor AtMYC2 positively regulates the expression of wound-responsive genes (i.e. *VSP*, *LOX* and *Thi2.1*) and represses other genes, including *PDF1.2*, *ChiB* and *HEL*. The JA and ethylene signals cooperate to activate the transcription factors ORA59, ERF1 and ORA37. ORA59 is the key regulator of the *PDF1.2*, *ChiB* and *HEL* genes in response to ethylene and JA, whereas the role of ERF1 in the regulation of these genes remains unclear (represented by a dashed arrow and a question mark). Conversely, ORA37 represses the induction of the *PDF1.2*, *ChiB* and *HEL* genes in response to JA and/or ethylene. ORA37 also enhances the JA-induced expression of *VSP* genes (circled plus), presumably by repressing the negative effect of ethylene operated through ERF1 (dashed bar line). The functions of other ORAs remain to be characterized.

In contrast, none of the putative JA-responsive target genes tested was found to be induced in the *ORA2*, *ORA4*, *ORA19*, *ORA31*, *ORA37* and *ORA44* lines. Except for ORA37, of which the role in JA signaling has been described in Chapter 4, the results can be interpreted to indicate that the ORA2, ORA4, ORA19, ORA31 and ORA44 proteins do not participate in JA signaling. However, it is more likely that these transcription factors regulate JA-responsive genes that were not tested in our screening. It is also possible that the activity of these ORAs requires JA-dependent post-translational modifications, such as protein phosphorylation or ubiquitination, or JA-dependent changes in sub-cellular localization or protein-protein interactions. If this is the case, the conducted screen would have been ineffective in the identification of target genes.

The aim of the thesis was to unravel the function of JA-responsive AP2/ERF-domain transcription factors, named ORAs, in the model plant species *Arabidopsis thaliana* by modulating their expression levels. The studies described in this thesis have led to the functional characterization of several ORAs, although the role of many ORAs in the JA signaling pathway remains unclear. The ORA33, ORA37, ORA47 and ORA59 proteins act as terminal components of the JA signal transduction pathway by regulating defense gene expression in response to JA. In addition, ORA59 and ORA37 not only integrate signals from JA but also from ethylene. ORA59 and ORA37 act antagonistically on the same subset of target genes, further increasing the complexity of the cross-talk between JA and ethylene. Figure 1 shows a model of the JA and ethylene signaling pathways and the role of ORAs, as well as other transcription factors, in these regulatory networks. In response to a specific stimulus, production of JA, as well as ethylene, leads to activation of several AP2/ERF-domain transcription factors, which, in turn, regulate the expression of specific genes resulting in defense. This work contributed to a better understanding of the molecular mode of action of JA in regulating gene expression in Arabidopsis. For instance, the results obtained with ORA59 caused significant changes in the existing view on the molecular components involved in JA-responsive gene expression and in the cross-talk between JA and ethylene (Figure 1; see also Figure 2 from chapter 1). The conclusions presented here essentially rely on data obtained from transcript analyses. In addition, based on several lines of evidence (discussed above), we speculate that follow-up research focused on ORA proteins and post-transcriptional regulation of ORAs might enlarge the present understanding of the role of ORAs in gene regulation and in JA signaling. Future challenges remain in unraveling the role of JA on the activity or stability of the ORA proteins and in identifying putative regulatory protein partners. Characterization of the transcription factors that regulate *ORA* gene expression is also of major importance to understand how JA orchestrates the complex defense response.

References

Atallah, **M** (2005). Jasmonate-responsive AP2-domain transcription factors in Arabidopsis. Ph.D. thesis, Leiden University, Leiden, The Netherlands.

Atallah, M., and Memelink, J. (2004). The role of the jasmonate signal transduction pathway in the response of plants to stress. In Encyclopedia of Plant & Crop Science, R.M. Goodman, ed. (New York: Marcel Dekker Inc.), pp. 1006-1009.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of *ETHYLENE-RESPONSE FACTOR1* in *Arabidopsis* confers resistance to several necrotrophic fungi. Plant J. **29**, 23-32.

Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J., and Manners, J.M. (2003). A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of Arabidopsis. Plant Physiol. **132**, 1020-1032.

Cheong, Y.H., Chang, H.-S., Gupta, R., Wang, X., Zhu, T., and Luan, S. (2002). Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. Plant Physiol. **129**, 661-677.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M. (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell **12**, 393-404.

Lorenzo, O., Piqueras, R., Sánchez-Serrano, J.J., and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15**, 165-178.

Lorenzo, O., Chico, J.M., Sánchez-Serrano, J.J., and Solano, R. (2004). *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell **16**, 1938-1950.

Menke, F.L.H., Champion, A., Kijne, J.W., and Memelink, J. (1999). A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J. **18**, 4455-4463.

Mikkelsen, M.D., Hansen, C.H., Wittstock, U., and Halkier, B.A. (2000). Cytochrome P450 CYP79B2 from *Arabidopsis* catalyzes the conversion of tryptophan to indole-3-acetaldoxime, a precursor of indole glucosinolates and indole-3-acetic acid. J. Biol. Chem. **275**, 33712-33717.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., and Ohme-Takagi, M. (2001). Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell **13**, 1959-1968.

Rojo, E., León, J., and Sánchez-Serrano, J.J. (1999). Cross-talk between wound signalling pathway determines local versus systemic gene expression in *Arabidopsis thaliana*. Plant J. **20**, 135-142.

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. **12**, 3703-3714.

Turner, J.G., Ellis, C., and Devoto, A. (2002). The jasmonate signal pathway. Plant Cell **14** (suppl.), S153-S164.

van der Fits, L., and Memelink, J. (2000). ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science **289**, 295-297.

van der Fits, L., and Memelink, J. (2001). The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J. **25**, 43-53.

Zuo, J., Niu, Q.-W., and Chua, N.-H. (2000). An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J. **24**, 265-273.