Analysis of gene expression in the outer cell layers of Arabidopsis roots during lateral root development

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Chapter 3

Regulation of the expression of *AIR1* and *AIR3* genes

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Abstract

AIR1A, AIR1B and *AIR3* are three auxin-responsive genes from *Arabidopsis* that are expressed in the outer cell layers of the root at sites of lateral root emergence. The expression of these genes is not directly regulated by auxin but mediated by a secondary messenger. In order to identify this secondary messenger we investigated the effects of known signaling factors/molecules on the expression of *AIR::GUS* reporter genes. Using two ethylene-insensitive mutant backgrounds, we found that the expression of *AIR1* and *AIR3* genes is ethylene-independent. Unexpectedly, methyl jasmonate (MeJA) led to increased *AIR1::GUS* expression with an expression pattern and intensity indistinguishable from that observed after auxin induction. The expression of *AIR1::GUS* and *AIR3::GUS* genes in mutants defective in auxin and jasmonate signaling was also studied. In *alf4-1*, a mutant impaired in lateral root formation, MeJA only poorly induced the expression of the *AIR1::GUS* gene. In the *slr-1* mutant however there was normal induction of the gene by MeJA. In the MeJA-insensitive mutant *coi1-1* the normal expression pattern of *AIR1* was observed but the enhanced expression of *AIR1* after auxin or MeJA stimulation was blocked. We propose that the auxin-induced expression of *AIR1* is mediated by MeJA, which activates the SCF^{COI1} machinery for the degradation of proteins repressing the expression of the *AIR1* gene. Unexpectedly, the *AIR3* gene turned out not to be responsive to MeJA. Thus the secondary signal triggering the auxin-induced expression of the *AIR3* gene remains unknown.

Introduction

Most studies concerning lateral root formation have been focused on the processes in the pericycle leading to the establishment of a new root meristem. However, other cell layers in the parental root are subject to changes during lateral root development. Neuteboom (2000) has shown that in the *Arabidopsis* root at least three genes, *AIR1A*, *AIR1B* and *AIR3* are expressed in the outer cell layers at sites of lateral root emergence. The genes *AIR1A* and *AIR1B* (hereafter referred to as *AIR1* unless indicated otherwise), and *AIR3* encode proteins that might be involved in structural weakening of tissues that are or will be penetrated by lateral root primordia. These *AIR* genes are late auxin-responsive (Neuteboom et al., 1999a) and their expression is hampered in mutants defective in lateral root formation, even after auxin induction (Chapter 2). These observations indicate that *AIR1* and *AIR3* gene expression is not directly triggered by auxin but via a secondary messenger. Most likely, this secondary messenger is a paracrine signal generated by auxin-activated pericycle cells (Chapter 2). Identification of this secondary signal is of vital importance to dissect the signaling pathway that leads to the activation of the *AIR1* and *AIR3* genes in the outer cell layers of the root. Purification of an unknown signaling compound from roots without any prior knowledge regarding its chemical nature is a very hard task. Therefore we first investigated several known signaling factors/molecules that might play a role in the *AIR* gene induction process based on their reported effects on plant cells and plant tissues.

Because plant hormones can fulfill signaling roles, several plant hormones other than auxins have been tested for their ability to trigger accumulation of *AIR* mRNAs as well as enhanced *AIR::GUS* expression (Chapter 2). However, gibberellic acid, abscisic acid, kinetin and salicylic acid (SA) were not able to activate the expression of *AIR*1 or *AIR3* genes. Also processes like cell division of pericycle cells (Chapter 2) or wounding were not able to activate promoter activity in *AIR1::GUS* and *AIR3::GUS* plants (Neuteboom, 2000).

Exogenously supplied auxin can stimulate ethylene biosynthesis by up-regulating the expression of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase in *Arabidopsis* (Yang and Hoffman 1984, review). Thus, it was possible that the auxin-induced expression of *AIR1* and *AIR3* genes was mediated at least in part

through increases in endogenous ethylene. However, Neuteboom et al. (1999a) did not find induced accumulation of *AIR1* or *AIR3* mRNA after treatment with the ethylene precursor ACC. We further confirmed these results by histochemical analysis of *AIR1::GUS* and *AIR3::GUS Arabidopsis* reporter lines treated with ACC (Chapter 2). Although these data demonstrated that it is unlikely that the *in planta* auxin-induced ethylene release is the mediating factor triggering *AIR* gene activation along the root, the data were not conclusive regarding the role of ethylene during normal lateral root formation in the absence of exogenously supplied auxin. For this reason, we re-investigated the effect of ethylene on the expression of *AIR1* and *AIR3* genes during lateral root formation using ethylene-insensitive mutants.

Reactive oxygen intermediates such as hydrogen peroxide (H_2O_2) and nitric oxide (NO) function as secondary signal messengers in the induction of defense genes in plants (Levine et al., 1994; Lamb and Dixon, 1997; Delledonne et al., 1998). NO has also been reported to be involved in the auxin response leading to adventitious root formation (Pagnussat et al., 2002). Another known signaling factor that might be relevant to *AIR* gene expression is acidification of the cell wall. Growing cell walls extend at acidic pH and this process is known as “acid growth”. A protein family known as expansins and hydroxyl radicals (OH) are involved in this process (Cosgrove, 1998; Schopfer, 2001). The effect of the above mentioned compounds on the expression of the *AIR1* and the *AIR3* genes was also studied.

Over the last years, jasmonate has emerged as a novel plant growth regulator and signal molecule. Jasmonic acid (JA) is a plant fatty acid derivative involved in the regulation of growth and development as well as in signaling of stress. Both jasmonate as well as its ester methyl jasmonate (MeJA) are found in plants and both exhibit biological activity (Creelman and Mullet, 1997; Creelman et al., 2002). During studies reported in this chapter, we found an intriguing effect of MeJA as this compound induced *AIR1* promoter activity with similar tissue specificity as auxin. Therefore, we focused on the study of MeJA as putative secondary signal leading to *AIR* gene expression. The type of interaction between auxin and MeJA regarding *AIR* gene expression was investigated. In addition, we studied A*IR1::GUS* and *AIR3::GUS* expression in mutants defective in auxin and jasmonate signaling. Based on the results obtained, we propose a model for the regulation of *AIR1* and *AIR3* gene activation.

Results

The role of ethylene in the expression of AIR genes

Two ethylene mutants, *etr1-1* and *ein2-1* (both considered as ethylene null-mutants) were used to investigate whether any kind of auxin-ethylene interaction could be leading to the expression of *AIR1* and *AIR3* genes. The *etr1-1* mutation is dominant and confers insensitivity to ethylene. *ETR1* acts early in the ethylene signal transduction pathway as an ethylene receptor. *ein2-1* is a recessive mutation conferring ethylene insensitivity. In *Arabidopsis*, ethylene signal propagation from *ETR1* to the nucleus requires *EIN2* (reviewed by Chang and Shockey, 1999).

Crosses were made between *AIR1::GUS* and *AIR3::GUS* reporter lines and the *etr1-1* and *ein2-1* mutants. Eight days old reporter-line seedlings carrying the *etr1-1* or the *ein2-1* mutation were treated with auxin (1 μM and 0.1 μM of 1NAA for *AIR1::GUS* and *AIR3::GUS*, respectively). In three separate experiments no effect of the *etr1-1* mutation on the induction of *AIR1* or *AIR3* genes by auxin was observed. In uninduced seedlings (control), the characteristic wild type expression pattern of the *AIR1* and *AIR3* genes (spots/rings) at sites of lateral root emergence was also observed in the *etr1-1* mutant. The same results were obtained for the *ein2-1* mutant background. Thus, the results obtained with the *etr1-1* and *ein2-1* mutants provide conclusive evidence that ethylene is neither involved in the normal (uninduced) nor in the induced expression of *AIR1* or *AIR3* genes in *Arabidopsis*.

Effect of H_2O_2, NO and acidification of the cell wall on AIR gene expression

The effect of reactive oxygen intermediates and acidification of the cell wall on the activation of *AIR1* and *AIR3* gene expression was assessed by histochemical analysis of GUS intensity in *AIR1::GUS* seedlings. The *AIR1::GUS* reporter line was used because it shows a stronger *GUS* expression pattern than the *AIR3::GUS* line.

Seedlings were incubated with 30 μM or 1mM H_2O_2. Since intracellular production of H_2O_2 can be provoked by addition of a yeast cell wall elicitor (Guo and Ohta, 1994), seedlings were also incubated with 0.1% (w/v) of yeast extract. Relatively high NO levels can be induced by addition of sodium-nitroprussiate (SNP)

to the medium (Delledonne et al., 1998). Therefore, to test the effect of NO on *AIR1::GUS* expression, seedlings were treated with 0.5 and 5 mM SNP. In plants, H_2O_2 interacts synergistically with NO to induce a hypersensitive disease-resistance response (Delledonne et al., 1998). To see whether a synergistic interaction between H_2O_2 and NO is needed for the induction of the *AIR* genes, we also tested H_2O_2 or yeast extract in combination with SNP. *AIR1::GUS* seedlings were treated with: 1 mM H_2O_2 + 0.5 mM SNP, 30 μM H_2O_2 + 0.5 mm SNP, and 0.1% yeast extract + 0.5 mM SNP.

Analysis of the GUS intensity obtained showed that none of the mentioned treatments induced or altered the characteristic expression pattern of the *AIR1::GUS* reporter gene. Thus our data do not support a role for reactive oxygen intermediates or NO, alone or in combination, in the up-regulation of *AIR1* gene expression.

The fungal toxin fusicoccin is known to induce acidification of the cell wall (Kutschera and Schopfer, 1985; Cosgrove, 1998). Addition of fusicoccin to final concentrations of 0.1 and 10 μM did not lead to altered GUS activity in *AIR1::GUS* plants, indicating that acidification of the cell wall is not the signal leading to enhanced *AIR* expression.

The physiological effectiveness of the range of concentrations used in the experiments described here has been reported previously (Lamb and Dixon, 1997; Menke et al., 1999b; Delledonne et al., 1998; Cosgrove et al., 1984).

The role of MeJA on AIR gene expression

The plant hormone MeJA was tested for its effect on *AIR1::GUS* expression. MeJA was added to final concentrations of 1, 10, 30 or 50 μM. Although none of these concentrations led to an increase in the number of lateral roots, *AIR1::GUS* expression was induced in the roots. At 1 μM the expression was relatively weak, but 10, 30 and 50 μM MeJA strongly induced GUS activity along the root with exception of the root meristem. The *AIR1* expression pattern was indistinguishable from the expression pattern previously observed after auxin induction.

AIR3::GUS plants were also treated with 1, 10, 30 and 50 μM MeJA. We found that the *GUS* expression in these plants did not change with respect to the control after any of the MeJA treatments tested. Only the typical rings around lateral roots

were observed, indicating that MeJA does not enhance *AIR3* gene expression and also does not change the normal (non-induced) expression pattern of this gene.

Interaction between auxin and MeJA in the induction of AIR gene expression

Since auxin and MeJA can induce the expression of the *AIR1* gene in a strikingly similar manner regarding pattern and intensity, we investigated the type of interaction of both hormones concerning *AIR1* gene expression. Ten days old *AIR1::GUS* seedlings were treated for 24 hours with 0, 0.01 or 0.1μM 1NAA alone and in combination with 0, 1 or 10μM of MeJA. *AIR3::GUS* seedlings were also tested. Earlier experiments in our laboratory had shown that the *AIR3* gene requires a ten-fold higher auxin concentration than the *AIR1* gene for optimal induction of the corresponding mRNA (Neuteboom et al., 1999a). Therefore, *AIR3::GUS* seedlings were treated with 0, 0.1 or 1μM 1NAA in the presence or absence of 0, 1 and 10 μM of MeJA. After incubation, the GUS intensity was analyzed. The results from two independent experiments are summarized in Table 1 and shown in Figure 1.

Table 1. Relative GUS intensity in roots of *AIR1::GUS* and *AIR3::GUS* seedlings after 1NAA, MeJA or 1NAA/MeJA induction.

MeJA	***AIR1::GUS***			***AIR3::GUS***		
	1NAA			1NAA		
	0	0.01 μM	0.1 μM	0	0.1 μM	1 μM
0	+[a]	+±	+++++	±[a]	+	+++
1 μM	++	++±	+++++	±[a]	±	+±
10 μM	+++	+++±	+++++	±[a]	±	+

The relative expression levels were quantified visually and indicated with + and ±. The range of expression along the root goes from highly expressed (+++++) to low *GUS* expression (±). In [a] , GUS activity was observed only at the sites of lateral root emergence, therefore +[a] or ±[a] indicate *GUS* intensity at these specific sites.

As can be deduced from the relative *GUS* expression shown by the *AIR1::GUS* reporter line (Table1), auxin and MeJA have an additive effect. Samples treated with 1 μM or 10μM MeJA combined with 0.01μM 1NAA, showed higher GUS activity than samples treated with MeJA or 1NAA alone at the mentioned

concentrations (Figure 1 does not very well show these relative differences). At 0.1μM 1NAA, the GUS activity was so high that further increase due to MeJA was no longer detectable (Table 1).

Contrary to its effect on *AIR1*, MeJA hampered the auxin induction of *AIR3* (Table 1). This negative effect of MeJA on the induction of the *AIR3* promoter by auxin could be most clearly observed by comparing the GUS intensity at 1μM 1NAA alone with the intensity at 1μM 1NAA + 10μM MeJA (Figure 1).

AIR gene expression in auxin mutants

The additive inducing effect of auxin and MeJA on the expression of *AIR1* prompted us to investigate the MeJA-induced expression of the *AIR1* gene in auxin-related mutant backgrounds defective in lateral root formation. To this end, *AIR1::GUS* plants carrying the *tir1-1*, *tir3-1*, *axr1-12*, *sur2-1*, *alf4-1* or *slr-1* mutations were used for induction experiments with MeJA (30 μM). Only in the *alf4-1* mutant background the induction of *AIR1::GUS* expression by MeJA was very much reduced (Figure 2a and 2b). In the *sur2-1* background, high *AIR1::GUS* expression was already observed along the root without induction (see Chapter 2) and after MeJA application extra GUS activity was observed. The other mutant backgrounds, including *slr-1* (Figure 2d), showed the same *GUS* expression as similarly treated wild type plants, thus a 'ringed' pattern in non-induced seedlings and strong blue staining along the root after MeJA induction.

We also investigated the effects of the above-mentioned mutant backgrounds on the expression of the *AIR3* gene after MeJA treatment. In the wild type, *AIR3::GUS* expression is not induced by MeJA and in the *tir1-1, tir3-1, axr1-12, sur2-1, slr-1* or *alf4-1* mutant backgrounds this phenotype was not altered.

AIR gene expression in a MeJA insensitive mutant

Our finding that MeJA induced the expression of the *AIR1* gene prompted us to investigate the effects of a mutation in the MeJA signaling pathway on the expression of the *AIR* genes. To investigate that, crosses were made between the *AIR1::GUS* and *AIR3::GUS* reporter lines and the *coi1-1* mutant. The *coi1-1* mutation defines an *Arabidopsis* gene required for a reaction to jasmonate. It is a recessive

mutation that confers MeJA insensitivity and male sterility (Feys et al., 1994). *coi1-1* has wild type responses to other hormones and accordingly we observed that upon incubation for 3 days in medium containing 1μM IAA, *coi1-1* seedlings formed lateral roots as abundantly as the wild type control.

For induction experiments, seedlings from the reporter lines carrying the *coi1-1* mutation were incubated in medium containing 1NAA or MeJA for 24 hours and subsequently stained for GUS activity. The *GUS* expression pattern was analyzed in at least three independent experiments; the results are summarized in Table 2. Without induction, the usual 'ringed' pattern of *AIR1::GUS* and *AIR3::GUS* gene expression was observed in the wild type and in the *coi1-1* background. However, after auxin or MeJA treatment the GUS intensity in *AIR1::GUS* plants carrying the *coi1-1* mutation was drastically diminished as compared to the GUS intensity observed in the wild type control. In these mutant plants, only the 'rings' and a faint blue staining in the elongation zone of the root was observed after these treatments, whereas wild type control plants showed a strong GUS staining all along the root (Figure 2a and 2c).

Figure 1. *AIR1::GUS* and *AIR3::GUS* expression after auxin/MeJA treatment. The concentrations of 1NAA (horizontally) and MeJA (vertically) are given in μM. For *AIR3::GUS*, only the results with 1 μM 1NAA alone, and in combination with 1 and 10 μM MeJA, are shown. For all treatments, 8 seedlings per well are shown.

Figure 2. *AIR1::GUS* and *AIR3::GUS* expression.
a. *AIR1::GUS* expression in the wild type after treatment with 30 μM MeJA.
b. *AIR1::GUS* expression in the *alf4* mutant after treatment with 30 μM MeJA.
c. *AIR1::GUS* expression in the *coi1-1* mutant after treatment with 30 μM MeJA.
d. *AIR1::GUS* expression in the *slr-1* mutant after treatment with 30 μM MeJA.
e. *AIR3::GUS* expression in the *coi1-1* mutant after treatment with 1 μM 1NAA.

Contrary to *AIR1*, the auxin-induced expression of *AIR3* was not inhibited in the *coi1-1* background (Figure 2e). After careful observation of the staining pattern which resulted from auxin induction we noticed a slightly higher GUS activity in the *AIR3::GUS* plants with a *coi1-1* background than in the wild type. This finding agrees with the data reported in Table 1 where it was shown that expression of the *AIR3* gene is repressed by MeJA. In order to find out whether MeJA negatively affects the auxin-induced expression of the *AIR3* gene, we used the same experimental set up described earlier for the detection of an additive or synergistic interaction between auxin and MeJA to analyze the expression of the *AIR3::GUS* gene in the *coi1-1* background. We observed that in the *coi1-1* mutant auxin alone or in combination with MeJA activated the *AIR3::GUS* reporter gene to the same extent. The "negative" effect of MeJA on the auxin induction of the *AIR3* gene, as observed in the wild type and shown in Table 1, thus disappeared in the MeJA-insensitive *coi1-1* seedlings.

MeJA-biosynthesis blockers

To explore the effect of endogenous concentrations of MeJA on the auxin-induced expression of the *AIR3* gene, we analyzed *AIR3::GUS* expression in seedlings treated with MeJA synthesis blockers. Diethyldithiocarbamic acid (DIECA) and SA can inhibit MeJA biosynthesis (Farmer et al., 1994; Peña-Cortés et al., 1993; Menke et al., 1999b). A line carrying the *DR5::GFP:GUS* gene, that can be used to specifically monitor auxin-activated cells (Ulmasov et al., 1997), was used as control.

Three days old *AIR3::GUS* seedlings were incubated for 3 days in medium containing 0.1 mM DIECA, 0.5 mM DIECA, 10 μM SA or 50 μM SA and subsequently treated with 1NAA (5μM). After these treatments the samples were stained for GUS activity. We observed that the GUS intensity in *AIR3::GUS* seedlings treated with MeJA synthesis inhibitors was higher than in the untreated controls (results not shown). Without auxin induction, DIECA-treated seedlings showed the characteristic *AIR3::GUS* expression pattern, although with slightly stronger intensity. In the *DR5::GUS:GFP* control, auxin but not MeJA enhanced the activity of the reporter gene and the same response was observed after these treatments in the presence of MeJA-biosynthesis inhibitors.

AIR1::GUS seedlings were treated similarly with DIECA and SA. Without auxin induction no significant differences in *AIR1::GUS* expression were observed between

DIECA- or SA-treated and untreated seedlings. After auxin induction, no major differences in GUS intensity were observed between DIECA-treated and untreated seedlings. However, seedlings treated with 50 μM SA showed a slightly weaker GUS activity than untreated seedlings after auxin induction (results not shown).

Although the results obtained using MeJA synthesis inhibitors should be interpreted with caution because these inhibitors may not be very specific (Menke et al., 1999b), they were in agreement with our expectations. A reduction of endogenous MeJA accumulation slightly enhanced the “normal” (non-induced) and the auxin-induced expression of the *AIR3* gene. On the contrary, inhibition of endogenous MeJA accumulation (slightly) reduced the auxin-induced expression of the *AIR1* gene.

Discussion

AIR1 and *AIR3* are auxin-responsive genes. However, the relatively slow type of induction kinetics of *AIR1* and *AIR3* mRNAs after auxin induction (Neuteboom et al., 1999a), the fact that applied auxin did not overcome the impaired *AIR1* and *AIR3* gene expression in the *alf4-1* mutant (Chapter 2), and the absence of known auxin-responsive elements in their promoters (Chapter 4) indicate that auxin itself can not be the direct stimulus triggering the expression of these genes. Thus, a secondary signal may mediate the auxin-induced expression of the *AIR1* and *AIR3* genes. In this chapter we focused on the identification of this putative secondary signal.

Ethylene, reactive oxigen species, NO and cell wall acidification are not involved in AIR gene expression

Given that auxin stimulates the production of ethylene, we extensively tested ethylene as a signal candidate for triggering the expression of the *AIR1* and *AIR3* genes. Previous mRNA expression studies and *GUS* expression analyses of *AIR1::GUS* and *AIR3::GUS* plants had shown that *AIR* gene expression was not increased after ACC treatment (Neuteboom et al., 1999a and 2000; Chapter 2). Here, we studied the expression of both *AIR* genes in two ethylene resistant mutant

backgrounds, *etr1-1* and *ein2-1*. We found no effect of these mutations, neither on the normal (non-induced) nor on the auxin or MeJA (data not shown) induced expression of *AIR1* and *AIR3* genes. Therefore, our results provided conclusive evidence that activation of *AIR* gene expression is independent of ethylene action.

The effects of known secondary signals, such as reactive oxygen intermediates and acidification of the cell wall (induced by fusicoccin), on the expression of the *AIR1::GUS* gene were also analyzed. However, we found that none of these treatments were able to enhance the *AIR1::GUS* expression, indicating that they do not mediate the auxin-induced expression of the *AIR* genes.

MeJA induces the expression of the AIR1 gene

Although the penetration of lateral roots through the overlying cell layers must cause substantial tension in those cells that have to be pushed apart by the emerging lateral root primordium, it was found that the application of wounds had no effect on *AIR* gene expression (Neuteboom et al., 1999a). It was therefore surprising that the wounding-related plant signaling compound MeJA induced *AIR1::GUS* expression. Seedlings treated with MeJA showed an *AIR1::GUS* expression pattern indistinguishable from the pattern obtained after auxin treatment. The type of interaction between auxin and MeJA regarding *AIR1* gene expression was investigated. We found that simultaneous addition of these two hormones had an additive effect on the expression of the *AIR1* gene. Contrary to *AIR1*, the expression of the *AIR3* gene was not enhanced by MeJA treatment.

To further investigate this apparent auxin-MeJA interaction regarding *AIR1* expression, we analyzed the expression of the *AIR1::GUS* reporter gene in several auxin-related mutants backgrounds after MeJA treatment. We found that neither the *tir1-1, tir3-1, axr1-12, sur2-1* nor *slr-1* mutation interfered with the induction of *AIR1::GUS* expression by MeJA. Thus, although the *slr-1* mutation did not interfere with the induction of *AIR1* expression by MeJA, it did prevent induction by auxin (Chapter 2). These results indicate that the auxin and the MeJA pathways that are responsible for enhanced *AIR1* gene expression are separated at *SLR1.* In contrast, the *alf4-1* mutation severely impaired both the auxin (Chapter 2) as well as the MeJA-induced expression of *AIR1*, thus the auxin and MeJA pathways may converge before or at *ALF4.*

MeJA is a candidate secondary signal involved in the expression of the AIR1 gene

When we studied *AIR1* expression in the MeJA insensitive mutant *coi1-1*, the normal expression pattern of *AIR1* was observed but the enhanced expression of *AIR1* after auxin or MeJA stimulation was blocked (Table 2). This indicates that although MeJA is not required for the basal pattern of *AIR1* expression (spots/rings), it seems to be required for the enhanced (induced) expression along the root. This implies that the signal mediating *AIR1* gene expression at sites where lateral root formation appears is different from the secondary signal that triggers *AIR1* gene expression along the roots as occurs after exogenous application of auxin. We hypothesize that MeJA mediates the enhanced (along the root) type of *AIR1* gene expression. The fact that the *coi1-1* mutant is not altered in its response to auxin (Feys et al., 1994) but shows impaired expression of the *AIR1* gene after exogenous auxin (or MeJA) induction supports our hypothesis.

Addition of MeJA to *alf4-1* seedlings did not lead to *AIR1* gene activation. Although these data suggest that MeJA is not be the secondary signal emanating from auxin-activated pericycle cells, one can not exclude that *alf4-1* mutants are insensitive to aspects of MeJA signaling as well.

How can MeJA mediate the auxin-induced expression of AIR1?

Substrate-specific protein degradation is an important mechanism of regulation of a wide variety of biological processes. This process is preceded by substrate ubiquitination and requires the activity of an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3). SCF complexes are the largest family of E3 ubiquitin-ligases and are composed of four subunits: SKP1, a cullin family member, a small RING finger protein (RBX1) and an F-box protein, which functions as degradation substrate receptor (Deshaies, 1999).

Accumulative evidence shows that the auxin and MeJA response involves the ubiquitin-mediated degradation of transcriptional regulators of auxin and MeJA responsive genes, respectively (Ward and Estelle, 2001; Devoto and Turner, 2003). Thus, cross-talk between the jasmonate and the auxin signal pathways might be mediated by interaction between the components of this degradatory pathway. In *Arabidopsis* the SCF^{TIR1} E3 ubiquitin-ligase plays a central role in the response to

auxin (Ward and Estelle, 2001). COI1, an F-box protein closely related to TIR1, appears to function by targeting yet-uncharacterized repressors of jasmonate-induced genes for removal by ubiquitination (Xie et al., 1998; Turner et al., 2002). It has been demonstrated that COI1 interacts, just like TIR1, with the proteins SKP1 and Cullin to form a SCF^{COI1} complex *in vivo* (Devoto et al., 2002; Xu et al., 2002). Auxin and jasmonate signaling share other components of the ubiquitin-mediated proteolysis pathway. Both the auxin and the MeJA response requires COP signalosome activity (Schwechheimer et al., 2001; Schwechheimer et al., 2002; Feng et al., 2003). The COP9 signalosome (CSN) is a protein-complex that was initially identified in plants as repressor of photomorphogenesis in the dark; later it was found to be involved also in several other developmental pathways in almost all eukaryotes (Serino and Deng, 2003). CSN interacts with the SCF complex, and several lines of evidence suggest that the CSN regulates the E3 ubiquitin-ligase activity of SCF complexes (Lyapina et al., 2001; Schwechheimer et al, 2001). It is also hypothesized that the CSN itself can associate with or be part of the proteosome (Serino and Deng, 2003). Thus, an integration of auxin- and jasmonate-signaling pathways may be achieved through the degradation of common target regulatory proteins. The MeJA insensitive mutant *coi1-1* shows impaired auxin- and MeJA-induced expression of the *AIR1* gene. We therefore propose that auxin, through MeJA, activates the degradation of negative regulators of *AIR1* by the CSN-SCF^{COI1} machinery leading to enhanced *AIR1* gene expression (Figure 3).

Little is known about SCF^{COI1} targets. By using a yeast two hybrid screen with COI1 as bait and coimmunoprecipitation experiments with epitope-tagged COI1, Devoto et al., (2002) isolated two potential substrates for COI1-mediated ubiquitination. A histone deacetylase RPD3b (reduced potassium dependency) and the small subunit of RUBISCO. Histone deacetylase functions to maintain the balance between acetylation and deacetylation of histones and forms an important mechanism in the regulation of gene transcription in eukaryotes. Histone deacetylation is believed to decrease accessibility of chromatin to the transcription machinery. Removal of RPD3b by COI1 may thus allow initiation of transcription of jasmonate responsive genes. The relation of RUBISCO as target for COI1 is that during senescence, the level of jasmonate in leaves increases while the expression of the small subunit of RUBISCO is reduced (Devoto and Turner, 2003).

A question rising from the model shown in Figure 3 is why the *axr1-12* mutation had no effect on the auxin- or MeJA–induced expression of *AIR1*. The AtCul1 (cullin) component of SFC^{COI1} is modified by RUB1 attachment which is AXR1 dependent (Xu et al., 2002). This modification is though to enhance the SCF E3 ubiquitylating activity (Hellmann and Estelle, 2002). Therefore, it is expected that a mutation in AXR1 results in a reduced jasmonate response and it does not. It has been found that *axr1-12* is a leaky mutation with respect to the modification of the AtCul1 subunit since low levels of modified AtCul1 are still found in the *axr1-12* mutant (Xu et al., 2002). The fact that the *axr1-12* mutation is leaky might provide an explanation for the unaltered *AIR1* expression in this mutant background. Another explanation could be gene redundancy for *AXR1*. In fact, the presence of a closely related gene in *Arabidopsis*, called *AXL1*, has been reported and it may partially complement *AXR1* (del Pozo et al., 2002).

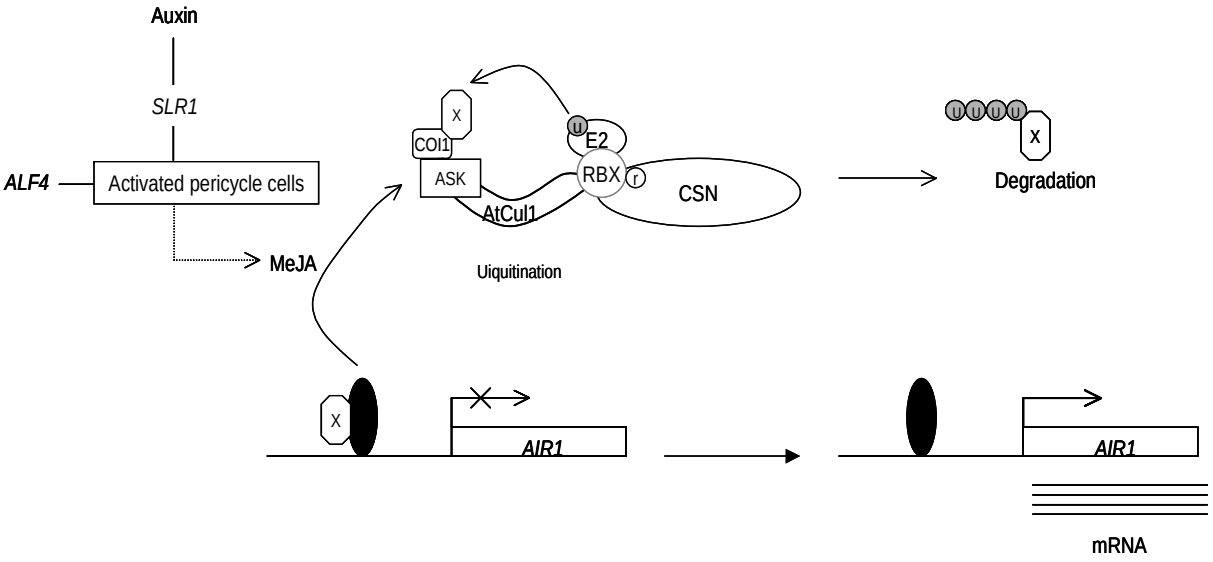

Figure 3. Model for auxin-induced expression of the *AIR1* gene.

The products of *ALF4* and *SLR1* are required to activate pericycle cells to divide and form lateral roots and therefore, are required for expression of the *AIR1* gene (Chapter 2). Activated pericycle cells generate a paracrine signal (MeJA) which induces ubiquitination and thereby degradation of a repressor protein (X) through the CSN-SCF^{COI1} complex. The protein X may repress the expression of *AIR1* by binding directly to the promoter, or by modifying the local chromatin (e.g. by deacetylation [not shown]), or by binding to an *AIR1* transcription factor (black oval). See text for additional details. Abbreviations: r: RUB1; u: ubiquitin; AtCul1: cullin; ASK: SKP1 homologue

The Auxin-induced expression of the AIR3 gene is affected by MeJA.

If MeJA is the secondary signal mediating the auxin-induced expression of *AIR1* (through *COI1*), it is certainly not the secondary signal that enhances the expression of the *AIR3* gene for the following reasons: (i) the expression of the *AIR3* gene is not induced by MeJA treatment and (ii), the auxin-induced expression of *AIR3* is not impaired in the *coi1-1* mutant background. After more careful analysis we found that MeJA had a negative influence on the auxin-induced expression of the *AIR3* gene. The latter observation was supported by results obtained in experiments where auxin and MeJA were applied together, and in experiments with the *coi1-1* mutant and with MeJA-biosynthesis blockers. The counteracting effects of jasmonate on the physiological action of auxin are well known but not yet understood. For instance, MeJA inhibits the auxin-regulated elongation of etiolated oat coleoptiles (Irving et al., 1999). The two vacuolar glycoprotein acid phosphatases (*VspA* and *VspB*) from soybean are differentially regulated by jasmonate and auxin during early stages of seedlings growth (Tang et al., 2001).

The *AIR1* and *AIR3* genes are expressed in the same tissues, during the same process and, induction of their expression is impaired in the *slr-1* and in the *alf4-1* mutant backgrounds (Chapter 2). These observations imply that the *AIR1* and *AIR3* genes share common regulatory pathways. We could propose that the auxin-induced expression of *AIR3* is regulated by a similar mechanism of degradation of regulators as proposed for the *AIR1* gene. However, the fact that the *coi1-1* mutation does not affect the auxin-induced expression of *AIR3* indicates that degradation of a putative *AIR3* regulator may occur via a different E3 ubiquitin-ligase complex. Supporting this assumption, a transcription factor NAC1 has been found to be involved in the regulation of expression of the *AIR3* gene (Xie et al., 2000). *NAC1* is an early auxin induced gene, which encodes a transcription activator with a conserved N-terminal domain that binds to DNA and a C-terminal activator domain which is characteristic for the NAC family of transcription factors. Plants overexpressing *NAC1* showed increased expression of *AIR3,* and more and longer lateral roots compared to control plants, while *NAC1* anti-sense plants showed the opposite phenotype (Xie et al., 2000). NAC1 activity is regulated by SINAT5, a RING-finger protein with ubiquitin E3 ligase activity, which promotes degradation of NAC1 by ubiquitination (Xie et al., 2002). However, as Xie and colleagues proposed, in

contrast to TIR1 (and in our “AIR1 model” to COI1), which activates auxin signaling by protein degradation, SINAT5 may attenuate the signal (and therefore *AIR3* expression) by targeting NAC1 for degradation.

Conclusions

The results presented in this study indicate that auxin application is a trigger that leads to enhanced *AIR1* and *AIR3* gene activation via a secondary messenger. We propose that MeJA is the secondary messenger leading to enhanced *AIR1* gene expression. However, the basal expression pattern of the *AIR1* gene (spots/rings) in the outer cell layers at sites of lateral root formation and emergence is not MeJA-dependent.

We hypothesize that MeJA activates the degradation of proteins repressing, directly or indirectly, the expression of the *AIR1* gene through the CSN-SCF^{COI1} complex. The secondary signal triggering the auxin-induced expression of *AIR3* is unknown. We assume that, in analogy with *AIR1*, the auxin-induced expression of *AIR3* is regulated by ubiquitin-mediated proteolysis of transcription factors but now including NAC1 and the ubiquitin-ligase SINAT5. That the expression patterns of *AIR1* and *AIR3* genes are very similar, but nevertheless brought about by different transcription factors is in line with the finding that the promoter regions of these genes seem not to share common regulatory elements (Chapter 4).

Material and Methods

Plant material

Arabidopsis plants homozygous for an *AIR1*A::GUS or *AIR3::GUS* construct in a Columbia background (Neuteboom et al., 2000) were used for all the experiments and for the crosses. The mutants *etr1-1* (Col-0) and *ein2-1* (Col-0), were provided by the Notthingham *Arabidopsis* Stock Center and the *coi1-1* (Col-0) mutant was kindly provided by Dr. John Turner (University of East Anglia, Norwich, UK). The

DR5::GUS:GFP line was kindly provided by Dr. Remko Offringa (Leiden University, The Netherlands)

AIR1A and *AIR3* reporter lines were crossed with *etr1-1* and with *ein2-1* mutants, in both directions, thus the mutants were used as male and as female. *AIR1::GUS* and *AIR3::GUS* plants carrying an *etr1-1* or *ein2-1* mutation were initially selected in the F2 population from the crosses by their kanamycin-resistant phenotype (selection marker of the *AIR::GUS* construct). The selected seedlings were further screened by their ethylene mutant phenotype like absence of the apical hook when germinated in the dark and resistance to ACC (10 μM). Since *etr1-1* is a dominant mutation, homozygous as well as heterozygous seedlings were used in the experiments.

Mutant *coi1-1* was used in the crosses as female and the reporter lines as pollen donor. In the F2, *coi1-1* mutants were selected by germinating seedlings on ½ MS medium supplemented with 30 μM MeJA. Four days after germination the difference between MeJA resistant (*coi1-1*) and sensitive (wild type) seedlings was clear. Wild type seedlings became brown and stopped growth after 2-3 days of germination while the *coi1-1* mutants remained green and grew normally. Four days after germination the resistant seedlings were transferred to MeJA-free medium for six days before induction.

The *AIR1A::GUS* and *AIR3::GUS* lines carrying the *tir1-1*, *tir3-1*, *axr1-12*, *sur2-1*, *slr-1* or *alf4-1* mutation are described in Chapter 2.

Induction experiments and GUS staining

All induction experiments were performed following the same procedure as described in Chapter 2. Inductions with MeJA alone or in combination were performed separately since this compound is highly volatile. A stock solution of MeJA was dissolved in DMSO; therefore a similar volume of DMSO was used in the controls. We never observed induction of *AIR1::GUS* or *AIR3::GUS* expression by DMSO at the final concentration used (0.1% v/v). Staining for GUS activity was performed as described in Chapter 2.

Treatments with MeJA-synthesis inhibitors

Three days old seedlings from the *AIR3::GUS* or the *AIR1A::GUS* line were incubated on plates containing 25 ml of solidified ½ MS medium without further additions (control) or containing 0.1 mM DIECA, 0.5 mM DIECA, 10 μM SA or 50 μM SA for 3 days. After this period, 5 ml of liquid ½ MS medium supplemented with 1NAA was carefully pipetted on the plates. Most of the liquid medium was quickly absorbed by the solid medium, resulting in a final concentration of 5 μM 1NAA. Plates were then incubated overnight at 21°C. After these treatments seedlings were staining for GUS activity.

Chemicals

Salicylic acid (SA) and nitroprusside (SNP) were obtained from Sigma (St Louis, MO, USA), yeast extract from Difco (Detroit, MI, USA), hydrogen peroxide (H_2O_2) from Merk (Darmstadt, Germany), MeJA from Bedoukian Research Inc. (Danbury, CT, USA) and DIECA from SIGMA (St Louis, MO, USA). Fusicoccin was a kind gif of Dr. Christa Testerink (TNO, Leiden, The Netherlands).