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

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# Chapter 2

# Signaling pathways involved in *AIR1* and *AIR3* gene expression

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## Abstract

The auxin-responsive genes *AIR1A, AIR1B* and *AIR3* from *Arabidopsis* are expressed in the outer cell layers of the parental root at specific sites of lateral root emergence. In this chapter we identified signaling pathways involved in the regulation of the expression of these genes. To this end, we checked the auxin-specific response of *AIR1* and *AIR3* genes. Furthermore, we analyzed the expression of these genes in mutant backgrounds defective in lateral root formation. We found that the genes are highly expressed in the *sur2-1* background at sites where abundant (adventitious) roots are being formed, showing that the expression is not strictly root specific but rather correlated with lateral and adventitious root formation. In *alf4-1* and *slr-1,* two mutants impaired in lateral root formation, the expression of *AIR1::GUS* and *AIR3::GUS* was impaired. In experiments with cell cycle inhibitors we found that *AIR1::GUS* and *AIR3::GUS* expression in the outer layers of the parental root is independent of cell division activity in the pericycle. Moreover, with the use of two marker genes highly expressed in dividing cells as reference, we have tried to identify the place and time point in which *AIR1* and *AIR3* genes are activated during lateral root primordium formation. We propose that a secondary signal emitted from auxin-activated pericycle cells triggers the expression of the *AIR1* and *AIR3* genes. Production of this secondary signal must involve *SLR1* and *ALF4*.

## Introduction

Lateral roots of higher vascular plants originate from the pericycle, the outermost layer of the vascular cylinder of the root (Charlton, 1996). Auxin and polar auxin transport from the shoot to the root and in the root from the root apex to the base play a key role in lateral root formation. This has been extensively demonstrated by physiological and genetic studies (Chapter 1). In the diarchic roots of *Arabidopsis*, lateral roots are initiated from pericycle cells located in files adjacent to a xylem pole. Upon treatment with exogenous auxin, additional lateral roots are initiated along the length of the primary root (Laskowski et al., 1995; Himanen et al., 2002). Several reports describing in detail the different stages of lateral root primordium formation have been published (Malamy and Benfey, 1997; Beeckman et al., 2001; Himanen et al., 2002; Dubrovsky et al., 2001). However, very little is known about the physiological processes involved in lateral root emergence. During lateral root primordium development and lateral root emergence, the outer cell layers of the parental root are pushed apart. It was generally thought that this was accomplished just by the action of the dividing cells of the newly developing lateral root. Surprisingly, Neuteboom et al. (1999b and 2000) isolated a group of auxin-responsive genes, *AIR1A, AIR1B* and *AIR3*, highly expressed in endodermis-, cortex- and epidermis cells at sites where lateral root primordia are being formed. Concomitant with the emergence of the lateral root primordium, the *AIR*-gene expressing cells form a ring surrounding the new lateral root. This shows that a new genetic program is induced in these cells which anticipates the arrival and penetration of the lateral root. Upon auxin treatment the *AIR1A, AIR1B* and *AIR3* genes are expressed along the whole root except for the root meristem, thus covering the whole area where lateral roots can be formed.

The *AIR1A* and *AIR1B* genes (hereafter referred to as *AIR1* unless indicated otherwise), encode proteins that share similarity with a large family of proteins consisting of a hydrophobic C-terminal part and a proline- or sometimes glycine-rich N-terminus. It has been proposed that such proteins reside in the plasma membrane, while their N-terminal sequences interact with components of the cell wall, thus linking the plasma membrane to the cell wall (Goodwin et al., 1996; Holk et al., 2002). The *AIR*1 genes are quite unique however, in that they only consist of the

hydrophobic region but lack the putative cell wall interacting N-terminus. According to this characteristic it is assumed that the AIR1 proteins might weaken the plasma membrane-cell wall connection by replacing family members containing the proline-rich N-terminus that fortify this connection (Neuteboom, 2000). *AIR3* encodes a putative subtilisin-type protease possessing all the characteristics of a secreted protein. It has been hypothesized that AIR3 is involved in digestion of components of the extracellular matrix (Neuteboom et al., 1999b), however, its protease activity has not been demonstrated yet. Thus, from the expression pattern and the type of proteins expected to be encoded by *AIR1* and *AIR3* it has been hypothesized that both genes are involved in weakening the connections between the cells of the parental root in the area through which the lateral roots will grow (Neuteboom, 2000).

In this chapter we try to learn more about the role of the *AIR1* and *AIR3* genes in lateral root formation and to get a better insight into the regulation of the expression of these genes. As a first step, we checked the auxin-specific response of the *AIR1* and *AIR3* genes. To this end, *Arabidopsis* plants carrying *AIR1::GUS* and *AIR3::GUS* constructs were induced with several plant hormones at different concentrations. After these treatments hystochemical analysis of the GUS activity was performed. Next, in order to further investigate the link between *AIR1* and *AIR3* expression and lateral root formation, we crossed *AIR::GUS* lines with mutants defective in lateral root formation. The pattern and intensity of GUS activity in the mutant backgrounds was studied before and after auxin treatment. Moreover, with the use of two marker genes highly expressed in dividing cells as reference, we tried to identify the place and the time point at which *AIR1* and *AIR3* expression is activated during lateral root formation. Since lateral roots are initiated in *Arabidopsis* by induction of cell division in specific pericycle cells (Chapter 1), we also investigated the effects of cell cycle inhibitors on the expression of *AIR1* and *AIR3* genes.

## Results

*AIR gene expression in response to auxin and other plant hormones*

Previous Northern blot analyses of *Arabidopsis* root cultures showed that *AIR1* and *AIR3* transcripts started to increase 4 hours after the addition of auxin, reaching the highest levels after 24 hours. Besides auxin, none of the other plant growth regulators tested (ethylene, gibberellic acid [GA], abscisic acid [ABA], cytokinin [kinetin]) was able to induce accumulation of *AIR1* or *AIR3* mRNA, and neither did salicylic acid (SA) (Neuteboom et al., 1999a). To analyze this more carefully we performed a histochemical analysis of *Arabidopsis* plants carrying the *AIR1::GUS* or the *AIR3::GUS* reporter gene, respectively. Five days old seedlings from these lines were treated overnight with 1-aminocyclopropane-1-carboxylic acid (ACC, 1-100 μM), GA3 (0.1-10 μM), ABA (1 and 10 μM), the cytokinin 6-benzylamino-purine (BAP, 1 and 10 μM) and SA (1-100 μM), respectively, and stained for GUS activity. We found that the basal *GUS* expression pattern did not change upon any of these treatments; only cells overlaying the places of lateral root primordia formation and cells surrounding new lateral roots exhibited GUS activity (results not shown). Roots from seedlings treated with 1NAA, IAA or 2,4-D (0.1-1 μM) stained blue, except for the root meristem (results not shown). These data thus corroborated and extended the results of Neuteboom et al. (1999a). Taken together, they clearly show (a), that none of the hormones tested altered the normal (uninduced) expression pattern of the *AIR1* and *AIR3* genes and (b), that enhanced *AIR1* and *AIR3* gene expression along the entire root was indeed specifically induced by auxins.

*Expression of AIR1 and AIR3 genes in auxin-mutants with reduced numbers of lateral roots*

Since *AIR1* and *AIR3* are auxin-inducible genes supposedly involved in the process of lateral root emergence, we wanted to investigate their expression pattern in auxin-related mutants of *Arabidopsis* which form reduced numbers of lateral roots. For this purpose, we crossed *AIR1* and *AIR3* promoter::*GUS* plants with the *tir1-1*, *tir3-1* and *axr1-12* mutants.

The *tir1-1* and *tir3-1* mutants (*tir* = transport inhibitor response) were isolated by their altered response to auxin transport inhibitors (Ruegger et al., 1997 and 1998). The *tir1-1* mutant is deficient in hypocotyl elongation and in the formation of lateral roots. Although auxin induces formation of new lateral roots in the *tir1-1* mutant, their number is less compared to the wild type (Ruegger et al., 1998). The *tir1-1* mutation is semi-dominant while the *tir3-1* mutation is recessive (Gil et al., 2001). *tir3-1* seedlings are strongly deficient in lateral root production but also here auxin is still able to induce formation of additional lateral roots. The *tir3-1* plants also display a reduction in apical dominance as well as a decreased elongation of siliques, pedicels, roots and inflorescences.

The *axr1* locus was identified by providing an auxin-resistant phenotype to *Arabidopsis* after mutation (Lincoln et al., 1990). The *axr1-12* mutation is recessive, and causes in the homozygous situation defects in the regulation of several early auxin responsive genes and has a drastic effect on plant morphology. Mutants have a short stature, wrinkled irregular-shaped leaves, reduced fertility and a reduced number of lateral roots (Lincoln et al., 1990; Timpte et al., 1995).

Seven days old *AIR1::GUS* and *AIR3::GUS* seedlings carrying the *tir1-1, tir3-1* or the *axr1-12* mutation were treated with 0.1 or 1 µM 1NAA. Because, 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), seedlings carrying the *AIR3::GUS* reporter gene were treated with ten-fold higher auxin concentrations than *AIR1::GUS* seedlings. The GUS activity was studied in at least three independent induction experiments. However, in none of them did we see an altered *GUS* expression pattern as compared to that seen in the wild type (Table 1). Without induction the usual rings of *GUS* expressing cells were observed around emerging lateral roots. After induction with auxin, blue staining was observed all along the root except for the root meristem. In addition, auxin treatment induced the proliferation of lateral roots in these mutants although in lower numbers than in the wild type (results not shown). The deficiency in auxin signaling in the *tir1-1, tir3-1* and *axr1-12* mutants therefore does not negatively affect the (auxin inducible) expression of the *AIR* genes. Apparently, expression of the *AIR* genes only indirectly correlates with the presence of auxin in the root. Rather the competence of cells in the pericycle to start a lateral root primordium may trigger the expression of *AIR* genes.

*Expression of AIR1 and AIR3 genes in the auxin overproducing sur2-1 mutant*

In order to study the expression of the *AIR1* and *AIR3* genes in mutants that form more lateral or adventitious roots due to elevated free auxin levels or enhanced auxin sensitivity the *sur2-1* mutant was used. The *sur2-1* is a recessive mutation leading to an increased concentration of free IAA (active form) compared to the wild type (Delarue et al., 1998). As a consequence of the high IAA concentration the mutant has a long hypocotyl, epinastic leaves and develops an excess of adventitious root primordia concomitant with disintegration of cortical and epidermal cell layers in the hypocotyl.

We crossed *AIR1::GUS* and *AIR3::GUS* lines with *sur2-1*. Seven days old *AIR1::GUS* and *AIR3::GUS* seedlings carrying the *sur2-1* mutation were treated with 0.1 and 1 μM 1NAA respectively and their *GUS* expression was analyzed. Without induction, high GUS activity was observed in that part of the hypocotyl where abundant adventitious roots and primordia were formed and where peeling of the cortical and epidermal tissue occurred (Figure 1e). *AIR1::GUS* and *AIR3::GUS* seedlings with a *sur2-1* background showed already high *GUS* expression along the main root without auxin treatment (Figure 1f). After 1NAA application, extra GUS activity was observed in these mutant lines. These results showed that *AIR1* and *AIR3* gene expression is indeed much higher in the overlaying tissues of lateral or adventitious root formation in the *sur2-1* mutant. The results also show that *AIR* gene expression is not strictly limited to the root, but rather responds to the formation of root primordial either in the root or in the hypocotyls.

*AIR1 and AIR3 gene expression in mutants with defective lateral root formation*

If expression of the *AIR* genes responds to the formation of (lateral) root primordia, a lack of expression would be expected in mutants defective in lateral root formation. Therefore, we crossed *AIR1::GUS* and *AIR3::GUS* plants with the *alf4-1* (aberrant lateral root formation) and the *slr-1* (solitary-root) mutants.

The *alf4-1* mutation is recessive and homozygous mutants do not form lateral roots. In this mutant the pericycle cells do not respond to auxin for division and lateral root formation, but *alf4-1* roots remain sensitive to auxin inhibition of elongation (Celenza et al., 1995). The *slr-1* mutation is dominant and results in agravitropy in the

mutant, which also shows defects in the initial divisions of pericycle cells during lateral root formation. The *slr-1* mutant has reduced sensitivity to auxin. In this mutant pericycle cells can occasionally make the first anticlinal divisions but since no further periclinal divisions occur *slr-1* mutants do not form lateral roots, not even in the presence of auxin (Fukaki et al., 2002).

The *alf4-1* mutants were identified in the F2 population from the crosses by incubating 7 days old seedlings in medium containing 1 μM IAA for 3 days followed by transfer to medium without auxin for four additional days. After this treatment *alf4-1* seedlings occasionally formed lateral roots, but only at the primary root tip, while wild type seedlings formed lateral roots all along the main root. The expression of the *AIR1::GUS* and *AIR3::GUS* genes was analyzed in the selected *alf4-1* mutants after treatment with 0.1 and 1 μM 1NAA, respectively. Without 1NAA treatment the typical *AIR1::GUS* expression pattern of rings/spots at places of lateral root emergence that was normally observed in the wild type did not occur in *alf4-1* (Figure 1a and 1b). After auxin treatment strong *GUS* expression along the root was observed in the wild type (Figure 1g), while in the *alf4-1* background this expression was drastically diminished (Figure 1c and 1d). As mentioned above, after prolonged incubation with auxin, *alf4-1* seedlings can form a few lateral roots close to the primary root meristem and, interestingly, in that zone *AIR1* and *AIR3* expression was observed in the mutant background. Similarly to *AIR1*, the expression of the *AIR3* gene was drastically affected by the mutation in the *ALF4* gene. No expression of the *AIR3* gene was observed in the *alf4-1* mutant background before auxin induction and only a faint blue staining in the elongation zone of the root after 1NAA treatment. These results show that a mutation in *ALF4* strongly affects the normal (non-induced) as well as the auxin-induced expression of *AIR1* and *AIR3* genes.

In the *slr-1* background no expression of the *AIR1::GUS* or *AIR3::GUS* gene was observed neither before nor after 1NAA treatment, with the exception of a faint blue staining in the elongation zone of the root and a few weak spots along the root (Figure 1h and 1i).

Thus, the study of *AIR1* and *AIR3* gene expression in the *alf4-1* and *slr-1* mutant backgrounds showed that the activation of both genes requires proper functioning of *SLR1* and *ALF4* genes and therefore the presence of a pericycle which is competent to react to auxin to form lateral roots.

*Is AIR gene expression triggered by cell divisions in the pericycle?*

Since *alf4-1* and *slr-1* are defective in lateral root primordium formation, we hypothesized that cell division activity in the pericycle could be the signal triggering the expression of *AIR1* and *AIR3* genes. In an attempt to find evidence for this assumption we examined the effect of cell division inhibitors on the *AIR1::GUS* and *AIR3::GUS* expression. As a positive control the cell-cycle marker line *cyc1At::GUS* was used (Ferreira et al., 1994). Five-day-old seedlings were pretreated for 24 hours with one of the four cell division inhibitors used: nocodazole, colchicine, hydroxyurea and aphidicoline. Nocodazole and colchicine block the transition from G2 to M, while hydroxyurea and aphidicoline prevent DNA synthesis and keep the cells in G1. After pre-incubation, the plants were treated with a final concentration of 0.1 or 1 μM 1NAA for *AIR1::GUS* and *AIR3::GUS*, respectively, in the presence of cell-division inhibitors and examined after 24 hours. We did not observe any alteration in the expression pattern of the *AIR1* or *AIR3* genes in the non-induced controls in the presence of the inhibitors (results not shown). This can be expected as lateral root primordia were already present on the seedling roots, and the cell cycle inhibitors prevented further root growth.

**Figure 1.** Expression of *AIR1::GUS* gene in the wild type and in mutant backgrounds. Roots from the wild type (**a**) and *alf4-1* (**b**) incubated in ½ MS. Roots from the wild type (**c**) and *alf4-1* (**d**) after treatment with 0.1 μM 1NAA. Hypocotyl of *sur2-1* (**e**) with abundant adventitious roots. *sur2-1* main root (**f**). In **e** and **f**, samples were not induced with auxin. Five days old wild type seedling induced with 0.1 μM 1NAA (**g**). Five days old *slr-1* seedling incubated in ½ MS (**h**) and after induction with 0.1 μM 1NAA (**i**). The same expression was observed in *AIR3*::*GUS* plants with *alf4-1*, *slr1* and *sur2-1* backgrounds respectively (not shown).

**Figure 2.** Expression of *DR5::GUS* (**a** and **d**), *AML::GFP* (**b** and **e**) and *AIR1::GUS* (**c** and **f**) genes during lateral root primordium formation. **a-c**, expression during early stages of lateral root primordium formation (without auxin induction). **d-f**, expression at a later stage (without auxin induction). The *AIR3::GUS* gene showed the same expression pattern as that of *AIR1::GUS* (not shown).

Also induction of *GUS* expression in the roots by auxin was completely normal (as in the controls) in the presence of the inhibitors (results not shown). The functionality of the cell cycle inhibitors however could be seen as they prevented the formation of new lateral root primordial after the treatment with auxin. In the absence of cell cycle inhibitors an abundance of lateral root primordia appeared on the seedlings roots after auxin induction. Similarly, the control line *cyc1At::GUS* treated with cell cycle inhibitors and induced with 1NAA showed a reduced number of lateral root primordia and therefore no additional *GUS* expression (such as spots in lateral root meristems) was observed (results not shown). All these observations indicate that the treatments with cell cycle inhibitors were indeed blocking cell division in the pericycle and the formation of new lateral roots. Thus, our results suggest that *AIR1* and *AIR3* expression in the outer layers of the parental root is independent of cell division activity in the pericycle.

Since apparently cell division in the pericycle is not the trigger of *AIR1* and *AIR3* gene expression, we wanted to pinpoint when en where these genes were activated. To this end we made use of two auxin-reporter genes: *DR5::GFP:GUS* and *AML::GFP:GUS* as reference. *DR5* is a synthetic promoter containing a tandem repeat of auxin-responsive elements and driving high expression levels in dividing cells (Ulmasov et al., 1997). The *AML* gene encodes a plant ribosomal protein and its expression is induced by auxin in the pericycle and is visible already during the first division of a single pericycle cell and later in the newly formed lateral root primordia (Weijers et al., 2001). Thus *DR5::GFP:GUS* and *AML::GUS:GFP* expression was used to assess auxin sensitivity during initial stages of lateral root formation and to see at which stage the *AIR1::GUS* and *AIR3::GUS* genes were activated.

Microscopic observation showed that *DR5::GFP:GUS*, *AML::GFP:GUS*, *AIR1::GUS* and *AIR3::GUS* genes are expressed during early stages of lateral root formation, even before a primordium structure can be distinguished (Figure 2a-c). We could not distinguish a clear temporal difference in the expression of *DR5::GFP:GUS*, *AML::GFP:GUS*, *AIR1::GUS* and *AIR3::GUS* genes. However, we observed that the expression pattern of *DR5::GFP:GUS* and *AML::GFP:GUS* do not overlap with the expression pattern of *AIR1::GUS* and *AIR3::GUS* genes. *DR5::GFP:GUS* and *AML::GFP:GUS* are strongly expressed in lateral root primordia whereas *AIR1::GUS* and *AIR3::GUS* are expressed in the cortex and epidermis cells overlaying these primordia (Figure 2). Thus, these observations together with the results obtained with

*alf4-1* and *slr-1* mutants agree with the idea that a secondary signal is probably emitted from underlying auxin-activated pericycle cells, which triggers the expression of *AIR* genes.

## Discussion

There is little controversy concerning the positive contribution of auxin and polar auxin transport to the formation of lateral roots. *AIR1* and *AIR3* are auxin-responsive genes probably involved in the process of lateral root emergence. Neuteboom et al. (1999a) showed that the *AIR1* and *AIR3* mRNA levels in lateral root cultures from *Arabidopsis* were increased after auxin induction and not after treatment with other hormones. In this study we confirmed these results by histochemical analysis of whole seedlings. Plants containing the *AIR1::GUS* and *AIR3::GUS* reporter construct showed increased GUS activity along the root only after treatment with auxin. Seedlings treated with ethylene (ACC), GA, ABA, the cytokinin BAP or SA only showed the basal (non-induced) expression pattern of *AIR1* and *AIR3* indicating that none of these hormones enhanced or altered the normal expression of these genes in *Arabidopsis*.

To find further links between auxin-induced lateral root formation and increased *AIR1* and *AIR3* gene expression, we studied the expression of both genes in six mutants with reported defects in auxin-induced lateral root formation. The results obtained from this study are summarized in Table 1.

We found that the expression of *AIR1* and *AIR3* in *tir1-1, tir3-1* and *axr1-12* mutant backgrounds was not detectably altered. In spite of the fact that we never observed differences in *GUS* expression between wild type and these mutant lines, we cannot exclude the possibility that small variations escaped detection. One of the reasons why *AIR* genes are apparently normally expressed in the *tir1-1, tir3-1* and *axr1-12* backgrounds could be that these three mutants are still able to form lateral roots upon auxin induction although in less number than in wild type (Ruegger et al., 1997 and 1998; Knee and Hangarter, 1996).

The *TIR1, TIR3*, and *AXR1* genes have been cloned. TIR1 is an F-box protein component of the $SCF^{TIR1}$ E3 ubiquitin-ligase complex implicated in the auxin response (Ruegger et al., 1998; Gray et al., 1999). AXR1 is a subunit of the

hetrodimeric RUB1/Nedd8 E1-activating enzyme that mediates the first step in the RUB1 modification of the cullin subunit of the $SCF^{TIR1}$ E3-complex (Leyser et al., 1993; del Pozo et al., 1998; del Pozo and Estelle, 1999). This modification may enhance the $SCF^{TIR1}$ E3 ubiquitylating activity (Hellmann and Estelle, 2002). Thus, TIR1 and AXR1 act in the same regulatory pathway of auxin action. Surprisingly, the auxin-induced expression of *AIR1* and *AIR3* seems not to be affected by mutation in either *TIR1* or *AXR1* indicating that an alternative pathway may mediate the auxin-induced expression of these genes. Alternatively, the unaltered *AIR1* and *AIR3* expression in the *tir1-1* and *axr1-12* mutant backgrounds could be due to redundancy. A gene closely related to *AXR1* called *AXL1* has been identified in *Arabidopsis* and this may partially complement the lack of *AXR1* function in the *axr1-12* mutant (del Pozo et al., 2002). In the case of *tir1-1*, other members of the large F-box family might compensate for the loss of *TIR1* function. In line with this possibility Zenser et al. (2001 and 2003) found no differences in the degradation rate of AUX/IAA proteins between wild type and *axr1-12* or *tir1-1* mutants after auxin induction. Without auxin induction the degradation rate of AUX/IAA proteins in *axr1-12* was slower than in the wild type.

**Table1**. Relative expression of *AIR1* and *AIR3* genes in wild type and in mutant backgrounds before (control) and after auxin (1NAA) induction.

| **Root phenotype** | **mutant** | ***AIR1*** | | ***AIR3*** | |
|---|---|---|---|---|---|
| | | **control** | **1NAA 0.1 μM** | **control** | **1NAA 1 μM** |
| wild type | | +[a] | +++ | ±[a] | ++ |
| Reduced number of lateral roots | *tir1-1* | +[a] | +++ | ±[a] | ++ |
| | *tir3-1* | +[a] | +++ | ±[a] | ++ |
| | *axr1-12* | +[a] | +++ | ±[a] | ++ |
| Overproduction of adventitious roots | *sur2-1* | +++ | ++++ | ++ | +++ |
| no lateral roots | *alf4-1* | - | - | - | - |
| | *slr-1* | - | - | - | - |

The relative expression levels were quantified visually. The range of *AIR1::GUS* or *AIR3::GUS* expression observed goes from highly expressed (++++), to weakly expressed (±) or not expressed (-). In [a] , GUS activity was observed only at the sites of lateral root emergence (spots/rings), therefore +[a] or ±[a] indicate *GUS* intensity at those specific sites.

The *tir3-1* mutation has been found to be allelic to *doc1*, a mutation altering the expression of light-regulated (*CAB*) genes (Gil et al., 2001). The *TIR3/DOC1* gene has been renamed *BIG* because of the extraordinary size of the protein product. BIG has similarity to a mammalian protein called calossin that contributes to vesicle traffic during synaptic signaling. In *Arabidopsis BIG* is required for normal auxin efflux and it has been found to interact synergistically with genes involved in either auxin response (e.g. *AXR1*) or transport (*PIN1, PID1*) (Gil et al., 2001). In our experiments the expression of *AIR1* and *AIR3* genes was unaltered in the *tir3-1* background. This observation can be attributed to the fact that the *tir3-1* mutant is competent to form lateral roots (although with lower efficiency than the wild type), and it responds to auxin application with the formation of additional lateral roots.

*AIR gene expression in the sur2 mutant*

*Arabidopsis* plants with mutations in the genes *sur2* and *sur1/rty/alf1* (Delarue et al., 1998; Boerjan et al., 1995; King et al., 1995; Celenza et al., 1995), display an abnormally copious proliferation of roots and contain elevated amounts of free and conjugated IAA. In the *sur2-1* mutant one of the IAA synthesis pathways is up regulated resulting in formation of abundant adventitious roots on the hypocotyl (Barlier et al., 2000; Bak et al., 2001). Adventitious roots as well as lateral roots originate from the pericycle in the vascular cylinder. Normally, the pericycle from the hypocotyl is “incompetent”, but it can be triggered to form adventitious roots by the addition of auxin (Ozawa et al., 1998; Konishi and Sugiyama, 2003).

In the wild type, *AIR* genes are expressed at places where occasional adventitious-roots are formed in de-etiolated wild type seedlings (not shown). The high expression of the *AIR1* and *AIR3* genes both in the root and hypocotyl of *sur2-1* seedlings was therefore expected as this can be attributed to the high internal auxin concentration and to the abundant formation of lateral and adventitious roots. From these observations we can conclude that *AIR* gene expression is not strictly root-specific, but instead linked with activated pericycle cells forming lateral or adventitious roots.

*The expression of AIR1 and AIR3 requires the proper function of ALF4 and SLR1 genes*

The *alf4-1* mutation blocks the initiation of lateral roots (Celenza et al., 1995). In this mutant the expression of *AIR1* and *AIR3* genes is severely reduced and induction of these genes by auxin is very much impaired. The expression of *AIR1* and *AIR3* is also impaired in the lateral root-defective mutant *slr-1*.

The *ALF4* gene has been cloned and encodes a large nuclear protein needed to maintain the pericycle in competent stage to divide and form lateral roots (DiDonato et al., 2004). The *ALF4* gene is expressed in most plant tissues including lateral root primordia and its expression is not directly regulated by auxin (DiDonato et al., 2004). *ALF4* however seems to be linked to auxin signaling leading to lateral root formation (DiDonato et al., 2004) and therefore, mutation of this gene must affect other genes, such as *AIR1* and *AIR3*, involved in processes associated with lateral root development.

The *SLR1* gene encodes the AUX/IAA14 protein (Fukaki et al., 2002). AUX/IAA proteins are short-lived nuclear proteins expected to act as repressors of genes responsible for mediating the various auxin responses (Abel et al., 1994). Auxin promotes the degradation of these repressor proteins through the ubiquitin-mediated proteolysis pathway leading to diverse downstream effects associated with the auxin response (Ward and Estelle, 2001; Zizamalova and Napier, 2003). The gain-of-function *slr-1* mutation caused a single amino acid change within the domain II, one of the four conserved domains (I-IV) of the AUX/IAA protein family which is required for the characteristic rapid degradation of these proteins (Fukaki et al., 2003). It is assumed that the *slr-1* mutation makes the repressor insensitive to auxin-induced degradation leading to a permanent block of lateral root formation. We found that the expression of *AIR1* and *AIR3,* two auxin-responsive genes, was repressed in the *slr-1* mutant. *AIR1* and *AIR3* gene expression therefore depends on de auxin-induced degradation of the SLR1 repressor.

In summary, the results obtained with the *alf4-1* and *slr-1* mutants clearly show that the regulation of *AIR1* and *AIR3* gene expression is linked to lateral root formation and is therefore, *SLR1*- and *ALF4*-dependent. The relatively slow type of induction kinetics of *AIR1* and *AIR3* mRNAs (earlier mentioned) indicate that auxin itself is not the direct stimulus leading to expression. The expression pattern of the

*AIR1* and *AIR3* genes in cells overlying sites of lateral root primordium formation also supports the idea of a secondary signal emanating from auxin-activated pericycle cells which regulates the expression of these genes. By experiments with cell division inhibitors we showed that cell division activity in the pericycle is not the signal leading to *AIR* gene activation.

## Materials and Methods

*Plant material*

*Arabidopsis* plants homozygous for an *AIR1*A or *AIR3* promoter-*GUS* fusion construct in a Columbia background (Neuteboom, 2000) were used for all the experiments and for the crosses. The mutants *tir1-1* (Col-0) and *tir3-1* (Colo-0) were kindly provided by Mark Estelle (Indiana University, USA); *alf4-1* (Col-0) by John Celenza (Boston University, USA); *sur2-1* (Ws-0) by Catherine Bellini (LBC, Versailles Cedex, France); *slr-1* (Col-0) by Masao Tasaka (NIST, Nara, Japan) and *axr1-12* (Col-0) by Remko Offringa (Leiden University, The Netherlands). Remko Offringa kindly provided the *DR5::GFP:GUS* and *AML::GFP:GUS Arabidopsis* lines (Weijers et al., 2001)

G*rowth conditions*

Seeds were surface sterilized by incubation for one minute in 70% (v/v) ethanol, 15 minutes in 1% hypochlorite solution supplemented with 0.01% Tween-20 and four rinses with sterilized water. Seeds were plated on vertically oriented plates containing agar-solidified ½ Murashige and Skoog (MS) medium. Plates containing the seeds were vernalized for four days at 4°C in the dark to promote synchronized germination. Seedlings were grown for five to seven days in vertical position at 21°C under 16 hr light (4000 lux) / 8 hr dark cycle unless indicated otherwise.

*Crosses and selection of mutant backgrounds in the F2*

*AIR1A::GUS* and *AIR3::GUS* reporter lines (Neuteboom LW., 2000) were crossed with *tir1-1*, *tir3-1*, *axr1-12, alf4-1, slr-1* and *sur2-1* mutants. Crosses were made in both directions, thus the mutants were used as male and as female with the exception of *alf4-1* and *sur2-1*. Since *alf4-1* is male sterile, it was used only as female. In the F2 population from the crosses, the kanamycin-resistance (Km-R) marker from the *AIR::GUS* construct and the mutant appearance were used as criteria to select plants for the analysis. The *tir1-1* mutant seedlings were distinguished by their deficiency in cell elongation. The hypocotyl of *tir1-1* seedlings does not elongate at high temperature (28°C) like the wild type does. Other phenotypic characteristics of *tir1-1* like elongation of the main root in the presence of 10 μM of 1-naphtylphtalamic acid (NPA) were less clear for the selection of mutants. *tir3-1* and *axr1-12* were selected by their small size, compact rosette and less lateral roots compared with the wild type. *alf4-1* seedlings showed a compact rosette and short hypocotyls and several of these seedlings were transferred to the greenhouse in order to confirm that they were indeed male sterile.

The *sur2-1* mutant contains a T-DNA (not linked to the mutation) with a kanamycin-resistance marker gene. Therefore, *sur2-1* was used as pollen donor in the crosses. In the F2, seedlings displaying the *sur2-1* mutant phenotype and expressing *GUS* were selected to get homozygous plants for induction analysis. Since the *sur2-1* mutant has a Wassilewskija (Ws) background and the reporter lines a Columbia (Col) background, *AIR1A::GUS* and *AIR3::GUS* plants were crosses with a Ws wild type and the F2 populations from these crosses were used as control in the experiments with *sur2-1*.

*Treatments and staining*

For the induction experiments, seven-day-old seedlings were transferred to multi-well Macroplates (Greiner) containing 4 ml of liquid ½ MS medium for the controls, or the same medium supplemented with a concentrated solution of a phytohormone not exceeding 40 µl to reach the final concentration. When compounds were dissolved in DMSO, a similar volume of DMSO was used in the

controls. Seedlings were incubated at 21°C (16 hr/ 8 hr light-dark cycle) for 16-24 hours.

For the treatments with cell cycle inhibitors, the ½ MS medium was supplemented with a small volume of a stock solution of nocodazole, colchicine, hydroxyurea or aphidicolin to reach a final concentration of 10 μg/ml, 100 μg/ml, 10 mM or 10 μg/ml, respectively, and incubated for 24 hour. After this treatment 1NAA was added to a final concentration of 0.1 μM and incubated for an additional 24 hours.

For localization of GUS activity, seedlings were rinsed with water and incubated in a solution of 50 mM sodium-phosphate pH 7.0, 10 mM EDTA, 1 mM K-ferricyanide, 1mM K-ferrocyanide, 0.05% (w/v) sarcosyl, 0.1% Triton X-100 and 1 mg/ml of X-gluc at 37°C for a few hours or overnight. Then, the samples were fixed with 70% (v/v) ethanol or with a 3:1 ethanol : acetic acid solution. For microscopic analysis, samples were cleared with a solution of 8:3:1 chloral hydrate : water : glycerol. A Zeiss axioplan II microscope equipped with differential interference contrast (DIC) optics was used.

For analysis of GFP fluorescence, seedlings were mounted in liquid ½ MS medium and viewed on a Zeiss Axioplan microscope equipped with a BIO-RAD MRC1024 confocal microscope. The 488 nm laser line from the Kt/Ar laser was used to excite GFP, and the fluorescent signal was detected through a 510 nm bandpass filter.

*Chemicals*

The compounds 1-naphthalene acetic acid (1NAA), gibberellic acid (GA3), abscisic acid (ABA), 6-bezylamino-purine (BAP) and salicylic acid were obtained from Sigma (St Louis, MO, USA). 1-aminocyclopropane-1-carboxylic acid (ACC) from Behring (La Jolla, CA, USA). Colchicine, aphidicoline, nocodazole and hydroxyurea were purchased from Sigma (St Louis, MO, USA).

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