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

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Chapter 1

General introduction

Lateral roots

Lateral roots are an important means for the plant to increase its absorptive area and the volume of substrate exploited. In *Arabidopsis* lateral roots originate in the pericycle, the outermost layer of the vascular cylinder, from a subset of founder cells located adjacent to the two xylem poles (Laskowski et al., 1995). The first morphological evidence related to lateral root initiation occurs in two adjacent founder cells within the same cell file, which undergo polarized asymmetric transverse divisions. Further radial expansion and subsequent periclinal divisions result in the formation of lateral root primordia of which the first one emerges 5 to 7 days after seed germination. An identical series of mitotic divisions also occurs in the two adjacent pericycle cell files, thus a total of three adjacent pericycle cell files located opposite to a xylem pole are involved in the formation of a lateral root primordium (Casimiro et al., 2001 and 2003). Malamy and Benfey (1997) made a detailed description of the anatomical events occurring during lateral root formation, events that they divided in eight defined developmental stages as shown in Figure 1.

Auxin and lateral root formation

The plant hormone auxin plays a central role in lateral root formation. This has been extensively demonstrated by physiological and genetic studies. Mutants containing elevated amounts of free and conjugated indole-3-acetic acid (IAA) like *sur2* and *sur1/rty/alf1* display an abnormally copious proliferation of roots (Delarue et al., 1998; Boerjan et al., 1995; King et al., 1995; Celenza et al., 1995).

Polar auxin transport from the shoot to the root apex and from the root apex to the site of lateral root initiation plays a key role in the regulation of hormone flux, thereby influencing lateral root development. Removal of apical tissues or application

of polar transport inhibitors has been shown to inhibit lateral root development (Reed et al., 1998; Casimiro et al., 2001). In addition, the *tir1-1* and *tir3-1* mutants (tir = transport inhibitor response) which were isolated by their altered response to auxin transport inhibitors, are deficient in formation of lateral roots (Ruegger et al., 1997 and 1998). The putative auxin import carrier AUX1 is likely involved in the mechanism of auxin polar transport as well. In *aux1* mutants the number of lateral roots that are induced by auxin is half that in the wild type (Swarup et al., 2001; Marchant et al., 2002).

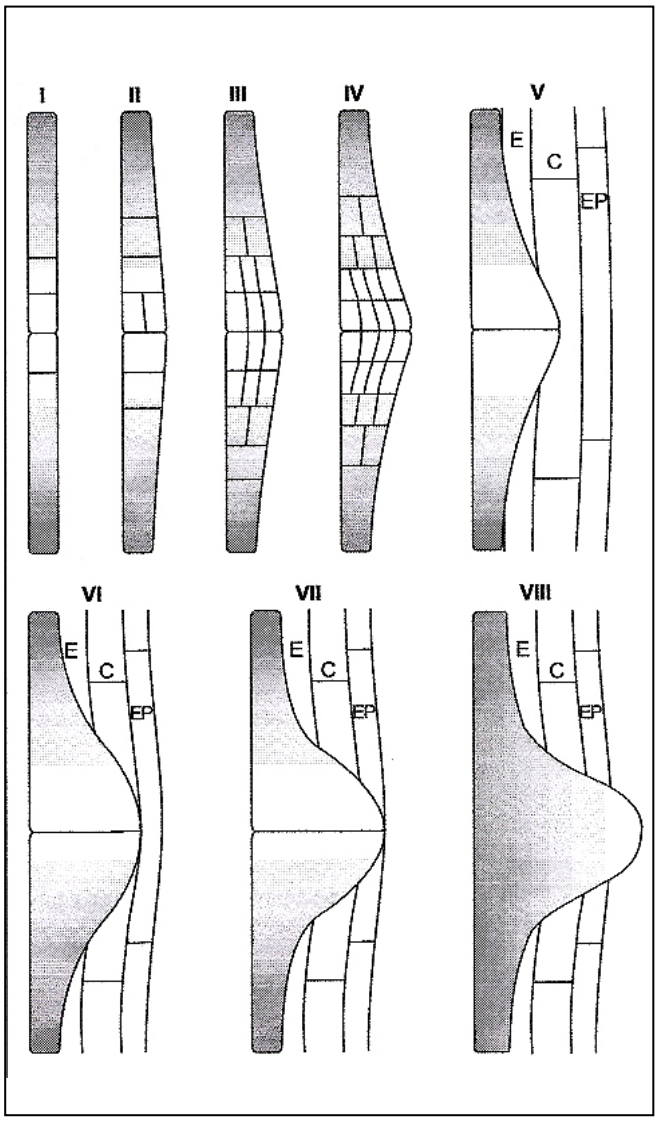

Figure 1. Schematic representation of the different developmental stages of lateral root formation in A*rabidopsis.* **Stage I**. Two adjacent founder pericycle cells undergo asymmetric transverse divisions. **Stage II**. A periclinal division occurs in one of the cells creating an inner layer and an outer layer. **Stages III and IV**. The inner and outer layers divide periclinally creating a total of four cell layers. Not all cells participate in these divisions thereby creating the dome-shape of the lateral root primordium (LRP). **Stage V**. Anticlinal and periclinal divisions occur. In addition, the internal layer enlarges pushing away the overlaying layers. At this stage the LRP penetrates the parental root cortex. **Stage VI**. Additional periclinal divisions occur and the LRP penetrates the parental epidermis. The LRP resembles a mature root tip. **Stage VII**. The LRP enlarges and is about to emerge. **Stage VIII**. The LRP emerges from the parent root, by expansion of the preexisting cells rather than by cell division. Abbreviations: C, cortex; E, endodermis; EP, epidermis. The figure is reprinted from Casimiro et al. (2003), with permission from Elsevier.

Recent *de novo* auxin biosynthesis measurements (Swarup et al., 2001; Bhalerao et al., 2002; Marchant et al., 2002) indicate that lateral root primordium development proceeds through three stages of dependence on auxin transport:

1. Initiation, during which discrete pericycle cells undergo a set of defined divisions. This step is dependent on the acropetal (from base to apex) and basipetal (from apex to base) polar auxin transport in the root.
2. Emergence, which requires shoot derived IAA.
3. Independence, the point at which the lateral root apex governs its own auxin balance and can synthesize its own IAA.

Despite considerable efforts, only two reported mutants have been isolated that are specifically affected in lateral root initiation, but not rescued by auxin application: *alf4-1* and *slr1*. The *ALF4* gene has been recently cloned and encodes a large nuclear protein with no similarities to proteins from other kingdoms. It is thought that the ALF4 protein is required to maintain the pericycle in a competent stage to divide and form lateral roots. The *ALF4* gene is expressed in most plant tissues including lateral root primordia and its expression is not regulated by auxin (DiDonato et al., 2004). The *SLR* gene encodes for the AUX/IAA14 protein (Fukaki et al., 2002). The auxin/indole-3-acetic acid (Aux/IAA) genes encode short-lived nuclear proteins that repress auxin-regulated gene expression through interaction with members of the ARF family of transcription factors (Abel et al., 1994; Kim et al., 1997). A specific domain within these repressor proteins (domain II) is responsible for the rapid, auxin-dependent, degradation of Aux/IAA proteins, which is executed by a specialized branch of the ubiquitin-proteasome pathway (reviewed by Zizamalova and Napier, 2003). The *slr1* mutation leads to a single amino acid change within the domain II that stabilizes the protein thus resulting in a gain-of-function mutation (Fukaki et al., 2002).

Regulation of lateral root initiation

It is a well-accepted idea that auxin activates pericycle cells to form lateral roots. However, it is not known why only specific pericycle cells (adjacent to the xylem pole) respond to a signal to become founder cells. In order to answer this question,

Beeckman et al. (2001) searched for differences between pericycle cells adjacent to a xylem pole and pericycle cells adjacent to a phloem pole in *Arabidopsis*. They observed that pericycle cells adjacent to the xylem pole do not remain in the G1 phase like their phloem counterparts, but proceed to the G2 phase. The authors proposed that xylem pericycle cells are more susceptible to lateral root initiation because these cells have completed DNA synthesis and remain at the phase that immediately precedes the M phase. However, not every pericycle cell opposite to a given xylem pole is involved in lateral root initiation indicating that additional mechanisms controlling the cell cycle are involved.

D-type cyclins play a prominent role in the regulation of cell division by their association with cyclin-dependent kinases (CDKs). The synthesis of D-type cyclins depends upon mitogenic signaling. It has been shown that a D-type-cyclin gene (*CYCD4;1*) is expressed in pericycle cells already at very early stages of lateral root initiation. At the time that the lateral root primordium is fully developed *CYCD4;1* expression becomes repressed (De Veylder et al., 1999). These results suggest that expression of D-type cyclins (including CYCD4) could be a key-limiting factor for lateral root formation.

Another study regarding the regulation of lateral root initiation described the involvement of the Kip-Related Protein2 (KRP2), a recently identified CDK-inhibitory protein (Himanen et al., 2002). *KRP2* transcription is down regulated by auxin; at low auxin concentrations the high level of *KRP2* expression seems to prevent lateral root initiation by blocking the G1-to-S transition. Moreover, *KRP2* transcripts accumulate in pericycle cells that are not implicated in lateral root initiation and transgenic plants overexpressing *KRP2* show more than 60% reduction in lateral root formation.

Other signaling pathways involved in lateral root development

Ethylene

The physiological interaction between auxin and ethylene in root development has been extensively studied and it is known that both hormones are endogenous regulators of root growth (Chang and Shockey, 1999 and references therein; Rahman et al., 2001). At least two kinds of interactions are well established: 1) exogenous auxin stimulates ethylene biosynthesis by induction of ACC synthase and 2), it has been

shown that ethylene inhibits the transport of auxin (Yang and Hoffman 1984; Suttle, 1988). Ethylene may also play a role in lateral root development because it inhibits root elongation with subsequent induction of lateral roots (Dolan, 1997).

More evidence of the cross talk between auxin and ethylene involving lateral root formation comes from studies in mutant backgrounds. For instance, *aux1* mutants have a reduced number of lateral roots compared to the wild type. Remarkably *aux1* mutations confer resistance both to auxin and ethylene (Bennett et at., 1996) and, more recently, Rahman et al. (2001) have shown that the resistance of *aux1-7* roots to ethylene disappears in the presence of auxin.

Nitrate

Nutrients such as nitrate have an important effect on lateral root development. In many plant species, exposure of the root to localized N sources results in an increased rate of lateral root proliferation (Forde, 2002). In *Arabidopsis*, this response consists of an increase in the elongation rate of lateral roots without an increase in lateral root number (Zhang and Forde, 1998 and 2000; Zhang et al., 1999). However, at high concentrations, nitrate leads to a systemic inhibition of lateral root elongation. Primary root growth is not affected by low or high nitrate conditions indicating that the effect on nitrate is specific for lateral roots (Zhang and Forde, 1998; Zhang et al., 1999).

Sucrose

Different effects of the sucrose-to-nitrate (C:N) ratio on root development have been reported. Zhang and Forde (1998 and 1999) observed that it was possible to alleviate the inhibitory effect of high nitrate concentrations on lateral root elongation by increasing the sucrose concentration in the medium. Malamy and Ryan (2001) found that a high C:N ratio represses lateral root formation in *Arabidopsis*, probably by impairing acropetal auxin transport. They isolated a mutant, *lin1* (lateral root initiation 1), which overcomes the repression of high sucrose-low nitrogen medium on lateral root formation.

ABA

The plant hormone abscisic acid (ABA) plays an important role in mediating the inhibitory effect of high nitrate on lateral root formation. The ABA insensitive mutants

abi4-1/2, abi5-1 and the ABA synthesis mutants *aba1-1, aba2-3/4*, and *aba3-2*, show a reduced inhibitory effect of nitrate on lateral root elongation (Signora et al., 2001). Furthermore, exogenous ABA mimics the inhibitory effect of high nitrate on lateral root elongation. Morphological analysis indicates that ABA inhibition occurs at a specific developmental stage, immediately after emergence of the lateral root primordium and before the activation of the lateral root meristem (De Smet et al., 2003). The ABA inhibition cannot be rescued by auxin (De Smet et al., 2003). These observations suggest that the ABA-inhibitory effect is auxin-independent.

Phosphate

The phosphate availability also can markedly influence root growth. Lopez-Bucio et al. (2002) found that *Arabidopsis* seedlings germinated in phosphate-deprived medium (<50 μM) had an increased lateral root density compared to seedlings germinated at high phosphate concentrations. In the latter case lateral root primordia were arrested just before emergence. The authors attributed the response of the roots of P-deprived seedlings to an increased auxin-sensitivity. In another study, Linkohr and co-workers (2002) found that lateral root elongation in *Arabidopsis* is restricted at high N and high P.

Lateral root primordia and overlaying tissues

Since lateral roots originate from the pericycle, they will have to penetrate the overlaying cell layers before emergence. Consequently, tissues outside the pericycle may influence lateral root development. It has been suggested that enzymes secreted by the growing lateral root primordium or by temporary structures derived from the overlaying endodermis (termed ‘Tasche’), hydrolyze cell wall components from outer cell tissues, thereby facilitating lateral root emergence (Charlton, 1996). Another hypothesis suggests that the outer cell layers of the parental root are separated from one another by physical pressure from the growing lateral root primordium. However, evidence supporting any of these suggestions is lacking. Neuteboom (2000) isolated a group of *Arabidopsis* genes that are specifically expressed in those cells overlaying the sites of lateral root primordia formation and emergence. It is assumed that the expression of these genes may play a role in lateral root emergence. Originally, the

genes were identified by means of a differential screening approach aimed at isolation of cDNA clones corresponding to mRNAs that are auxin-inducible in root cultures. Four cDNA clones were isolated, *AIR1, AIR3, AIR9* and *AIR12* (AIR for Auxin-Induced in Root cultures). Sequence analysis revealed that these four *AIR* cDNA clones encoded putative extracellular and membrane-associated proteins (Neuteboom et al., 1999 a and b). Northern blot analysis revealed that accumulation of mRNA transcripts of these genes started between 4 and 8 hours after auxin treatment.

Sequence analysis showed that AIR12 (corresponding to the *Arabidopsis* locus At3g07390) has so far no significant homology to any other available sequence. AIR9 (At2g34680) possesses a glycosyl hydrolase motif and leucine-rich repeat domains. The predicted AIR1 protein (At4g12550) consist of a N-terminal signal peptide and a hydrophobic, presumably membrane-bound C-terminus (Neuteboom et al., 1999a). However, AIR1 lacks the characteristic proline- or glycine-rich region located between the signal peptide and the C-terminal found in homologous proteins. The predicted AIR3 protein possesses all the characteristics of a subtilisin-like serine protease, which is believed to be active extracellularly.

Neuteboom et al. (1999) studied the *AIR1* and *AIR3* cDNA clones in more detail. It was found that only (active) auxins were able to induce accumulation of mRNAs corresponding to these clones. Treatment with other plant hormones such as gibberelic acid (GA_3), abscisic acid (ABA), kinetin, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and salicylic acid (SA) did not induce *AIR1* or *AIR3* mRNA.

AIR1 genes

Two highly homologous genes corresponding to the *AIR1* cDNA, termed *AIR1A* and *AIR1B* were isolated (Neuteboom, 2000). The predicted AIR1A and AIR1B proteins (111 and 108 amino acids respectively) possess a signal peptide at the N-terminus and a hydrophobic C-terminus and lack the characteristic proline- or glycine-rich region found in related proteins (Figure 2). Although AIR1A and AIR1B do not possess the characteristic proline-rich N-terminus, they show high homology in the C-terminal part with several proline-rich and glycine-rich proteins (PRPs and GRPs respectively) and with hydroxyproline-rich glycoproteins or extensins. Since this C-terminal region contains a conserved CLCT amino acid sequence, Neuteboom et al.

(1999a) renamed this family “CLCT proteins”. It has been proposed that the repetitive proline-rich or glycine-rich domains make cross-links with cell wall components, in this way coupling plasma membrane and cell wall (Deutch and Winicov, 1995). PRPs, GRPs and extensins have been associated with cell-type-specific wall structure determination during plant development (Fowler et al., 1999), and with defense reactions against physical damage and pathogen infection (Showalter, 1993). Their expression has also been closely associated with cells that eventually become lignified like protoxylem elements (Carpita and Gibeaut, 1993) and with emerging lateral roots (Vera et al., 1994), thus, where reinforcement of the cell wall is required to resist the mechanical pressure.

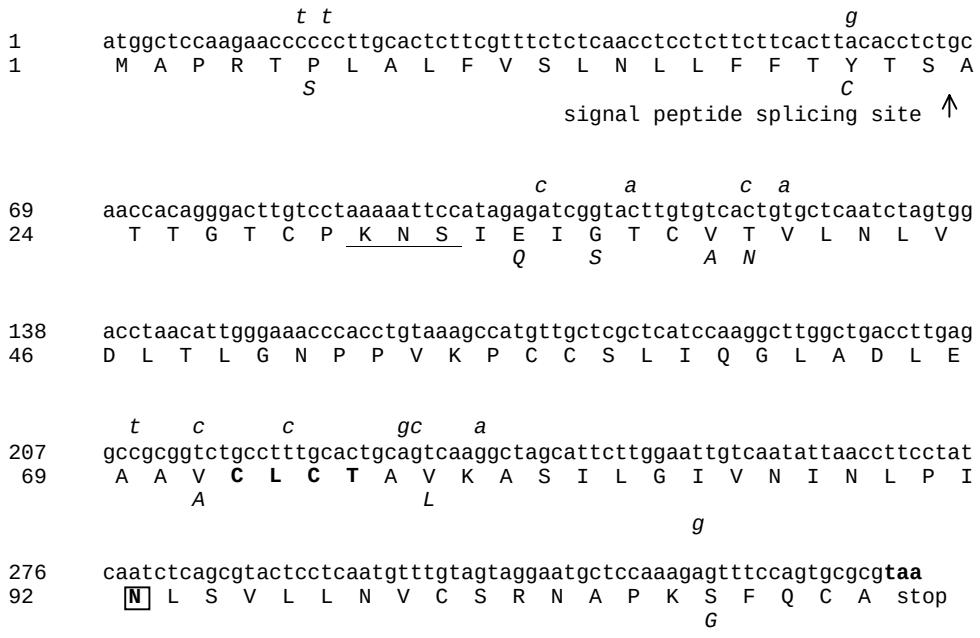

Figure 2. Nucleotide and deduced amino acid sequence of *AIR1A* and *AIR1B* coding regions.
The differences between *AIR1A* and *AIR1B* are indicated in italics; above the sequence, the differences of *AIR1B* with respect to *AIR1A* at the nucleotide level, below the amino acid sequence, the difference of AIR1B with respect to AIR1A at amino acid level. The arrow indicates the putative signal peptide splicing site of the protein. The three underlined amino acids are not present in AIR1B. The conserved CLCT amino acid sequence found in related proteins are indicated in bold face. A putative N-glycosylation site is boxed.

Arabidopsis transgenic plants carrying *AIR1A* and *AIR1B* promoter-reporter gene fusions showed that these genes are expressed in the epidermal, cortical and

endodermal cells of the parental root around the site of lateral root emergence (Neuteboom, 2000). The expression patterns of the *AIR1A* and *AIR1B* genes are identical. The proline-less proteins encoded by these genes and their expression pattern indicate that AIR1A and AIR1B proteins may weaken plasma membrane-cell wall connections by competing with their proline-rich homologues facilitating in this way lateral root emergence.

AIR3 gene

AIR3 is a single copy gene encoding a 772 amino acid protein belonging to the family of plant subtilisin-like serine proteases. The catalytic triad of the amino acids aspartic acid (D), histidine (H) and serine (S), together with the substrate-binding site (N), typical for this type of proteases, were found in the deduced amino acid sequence of the *AIR3* gene (figure 3) (Neuteboom et al., 1999b).

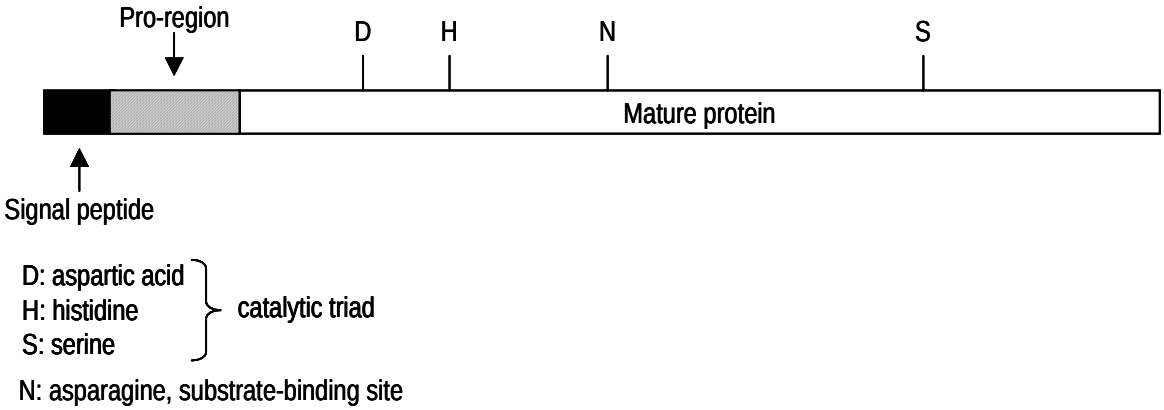

Figure 3. Schematic representation of the structure of the AIR3 protein.

In plants little is known about subtilase substrates. However, the high similarity in the catalytic domains and substrate binding sites indicates that they share enzymatic properties with mammalian subtilisins, which are involved in the cleavage of prohormones and proproteins at specific sites (reviewed by Bogacheva, 1999). All plant subtilisin-like proteases including AIR3 are believed to be active extracellularly.

Expression analysis showed that *AIR3* has almost the same expression pattern as *AIR1A* and *AIR1B* genes: in the outer cell layers of the parental root around lateral

roots and at sites where lateral roots are about to emerge. Upon auxin induction, *AIR3* expression increases along the length of the root except for the root meristem (Neuteboom et al., 1999b). The nature of the protein encoded by *AIR3*, its expected localization and its expression pattern suggest that AIR3 digests structural proteins in the extracellular matrix in order to weaken the tissue and facilitate lateral root emergence.

Outline of this thesis

The aim of this thesis was the further study of the *AIR1A*, *AIR1B* and *AIR3* genes and thereby to identify signaling pathways regulating the expression of these genes. Furthermore, we wanted to gain evidence of the role the *AIR1A, AIR1B* (hereafter referred to as *AIR1* unless indicated otherwise) and *AIR3* genes on lateral root development.

In **Chapter 2**, the auxin-specific response of the *AIR1* and *AIR3* genes in *Arabidopsis* plants carrying *AIR1* and *AIR3* promoter-*GUS* constructs (*AIR1::GUS* and *AIR3::GUS* lines) was studied. The expression of *AIR1* and *AIR3* genes in mutant backgrounds defective in lateral root formation was also investigated. Furthermore, experiments with cell cycle inhibitors were carried out in order to investigate whether expression of the *AIR* genes is dependent of cell division activity in the pericycle or not. Results from these analyses indicated that auxin itself is not the signal leading to the expression of AIR1 and AIR3 genes.

In **Chapter 3** the attention is focused on the identification of a secondary signal triggering the expression of the *AIR* genes. Known signaling factors were tested for their effect on the expression of the *AIR1::GUS* reporter gene. A putative effect of ethylene on the expression of *AIR1* and *AIR3* genes was investigated by analyzing the expression of these genes in two ethylene-insensitive mutant backgrounds. MeJA was identified as the most promising candidate as a secondary signal. Therefore, the interaction between auxin and MeJA regarding *AIR* gene expression was investigated. The *AIR1::GUS* and *AIR3::GUS* expression in the MeJA-insensitive *coi1-1* mutant background was also studied in this chapter. The results of these studies and those of Chapter 2 have led to the proposition of a model for the auxin-induced expression of the *AIR1* and *AIR3* genes.

In **Chapter 4** the 5'-flanking sequences of the *AIR1A* and *AIR1B* genes were analyzed. It was found that the *AIR1A* and *AIR1B* promoters possess highly homologous regions. Since the conservation of the sequences within these regions could be an indication of the importance of these sequences, these regions were studied by promoter-deletion::*GUS* analysis. The 5'-flanking sequence of the *AIR3* gene was also analyzed in this chapter. The shortest *AIR3* 5'-flanking sequence conferring the characteristic *AIR3* expression pattern was identified. In the second part of this chapter the position of *AIR1A, AIR1B* and *AIR3* in the *Arabidopsis* genome was determined and the surrounding sequences identified.

In **Chapter 5** we focused on the function of the *AIR3* gene. To this end *Arabidopsis* lines constitutively expressing *AIR3* under the control of the CaMV *35S* promoter were generated and an *AIR3*-knockout mutant line was isolated. The *35S::AIR3* plants showed a marked increase of lateral root lengths while primary root lengths and lateral root densities were unaffected. This phenotype is very similar to the phenotype seen in *Arabidopsis* after local nitrate stimulation. The possible role of *AIR3* in regulating lateral root growth and its relation with auxin and NO_3^- signaling was investigated and discussed. A model for the mode of action of *AIR3* is proposed.

In **Chapter 6** all the results obtained in Chapters 2 to 5 are summarized and discussed.