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

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Chapter 6

Summary and general conclusions

Branching of roots starts with the formation of new root apical meristems in the pericycle of the parental root, a process which is most likely controlled by the plant hormone auxin. Subsequently, lateral roots emerge from the pericycle through the endodermis, cortex and the epidermis. The process of lateral root formation itself has been studied extensively in a variety of plant species, in particular in *Arabidopsis*. However, whether and how cells in the outer cell layers react upon the formation of a lateral root primordium underneath is poorly understood. The identification of *AIR1A, AIR1B* and *AIR3*, three auxin-responsive genes from *Arabidopsis* which are expressed specifically in the outer cell layers of the parental root at sites of lateral root formation (Neuteboom, 2000), has demonstrated the existence of an interaction between lateral root primordia and overlaying cell layers. After auxin application, a treatment inducing abundant lateral root formation, the three *AIR* genes are expressed along the entire root, but still only in the outer tissue layers.

AIR1A and *AIR1B* are two highly homologous genes which encode proteins that are putatively associated with the plasma membrane (Neuteboom, 2000). They have strong similarity to proline- or glycine-rich proteins (Neuteboom et al., 1999a). It has been proposed that the proline- or glycine-rich regions of proteins related to AIR1A and AIR1B make cross-links with cell wall components coupling in this way plasma membrane and cell wall (Goodwin et al., 1996; Holk et al., 2002). AIR1A and AIR1B lack the proline- or glycine-rich region. Therefore, it has been hypothesized that accumulation of these proteins will result in weakening of plasma membrane-cell wall connections facilitating lateral root emergence (Neuteboom, 2000).

The protein encoded by *AIR3* possesses all the characteristics of a subtilisin-like serine protease. As proposed for plant subtilisin-like proteases, also AIR3 is believed to be active extracellularly. For this reason it has been assumed that AIR3 digests

components of the cell wall, thereby also facilitating lateral root emergence (Neuteboom et al., 1999b).

The research described in this thesis pursued two main goals: at the one hand to identify components of the signal transduction pathway regulating the expression of the *AIR1A, AIR1B* and *AIR3* genes and, at the other hand, to gain knowledge about the role of these genes during lateral root development. As for the first goal, the expression of the *AIR1::GUS* and *AIR3::GUS* reporter genes was studied in mutant backgrounds with a different reaction upon auxin treatment and/or with defects in lateral root formation (Chapter 2). The auxin-related mutants *tir1-1*, *tir3-1*, and *axr1-12*, show a relatively low number of lateral roots compared to the wild type (Ruegger et al., 1998; Gil et al., 2001; Lincoln et al., 1990; Timpte et al., 1995). In these mutant backgrounds no significant changes regarding the auxin-induced or the non-induced expression of the *AIR1* and *AIR3* genes were observed. In *alf4-1* and *slr-1*, two mutants completely impaired in lateral root formation (Celenza et al., 1995; Fukaki et al., 2002), the *AIR1* and *AIR3* genes were not expressed, not even after auxin treatment. On the contrary in the *sur2-1* background, a mutant with copious adventitious roots (Delarue et al., 1998), strong *AIR* directed GUS activity was observed in the outer cell layers of the root system and also in the hypocotyl at sites where adventitious roots were formed. The results obtained thus provide genetic evidence for an intimate link between lateral and adventitious root formation and the expression of *AIR1* and *AIR3* genes. In addition, it can be concluded that activation of the *AIR1* and *AIR3* gene is S*LR1* and *ALF4* dependent and therefore requires the presence of a pericycle which is competent to react to auxin and to form lateral roots.

The relatively slow type of induction kinetics of *AIR1* and *AIR3* mRNAs after auxin treatment (Neuteboom et al., 1999a) and the absence of auxin-responsive elements in their promoters (Chapter 4) were in line with the idea that auxin itself was not the stimulus for triggering the expression of these genes. Chapter 3 focuses on the identification of this putative secondary signal mediating the auxin-induced expression of the *AIR1* and *AIR3* genes. Since auxin triggers cell division activity in the pericycle it could be postulated that dividing cells generated a secondary signal. However, experiments with cell division inhibitors showed that cell division itself was not the signal leading to *AIR* gene activation. Because hormones can fulfill signaling roles, several plant hormones other than auxin were tested for their ability to trigger accumulation of *AIR1* mRNAs and activation of the *AIR::GUS* reporter genes. It was

found that gibberellic acid, abscisic acid, kinetin and salicylic acid (SA) were not able to activate the expression of the *AIR1* or *AIR3* genes. Another possible secondary signal was ethylene since exogenously supplied auxin stimulates ethylene biosynthesis in *Arabidopsis* (Yang and Hoffman, 1984). However, treatment with the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) did not induce accumulation of *AIR1* or *AIR3* mRNAs (Neuteboom et al., 1999a). These results were confirmed by histochemical analysis of *AIR1::GUS* and *AIR3::GUS* reporter lines treated with ACC and by expression analysis using the ethylene mutants *etr1-1* and *ein2-1*. From all these results we could conclude that the expression of *AIR1* and *AIR3* genes was independent of ethylene signaling. Other known secondary signals tested were reactive oxygen intermediates (H_2O_2 and NO) and acidification of the cell wall. Analysis of the GUS intensity obtained in the reporter lines showed that none of these signals were able to induce or alter the characteristic expression pattern of *AIR1* or *AIR3* genes. Methyl jasmonate (MeJA) was also tested for its effect on *AIR::GUS* expression. It was found that MeJA induced the expression of the *AIR1* gene with a pattern and intensity indistinguishable from that observed after auxin treatment. The expression of the *AIR1* gene was analyzed in the MeJA insensitive mutant *coi1-1*. In this mutant background the usual 'ring' pattern of *AIR1::GUS* expression was observed but the enhanced expression along the root after auxin induction was absent. This implies that the signal mediating the 'uninduced pattern' of *AIR1* expression is different from the signal triggering the enhanced expression pattern of this gene. It was therefore hypothesized that MeJA mediates the enhanced type of *AIR1* expression.

Recent papers show that auxin and MeJA responses involve the ubiquitin-mediated degradation of transcriptional regulators of auxin and MeJA responsive genes (Ward and Estelle, 2001; Devoto and Turner, 2003). The *COI1* gene encodes an F-box protein (Xie et al., 1998; Turner et al., 2002). The F-box proteins are components of the SCF ubiquitin-protein ligase (E3) complexes, in which they function as specific receptors targeting proteins to ubiquitin-mediated proteolysis (Ward and Estelle, 2001; Hellmann and Estelle, 2002). The MeJA insensitive mutant *coi1-1* was shown to be impaired in auxin- and MeJA-induced expression of the *AIR1* gene (Chapter 3). This clearly indicated that the SCF^{COI1} complex plays a key role in the regulation of the expression of this gene. It was therefore proposed that MeJA mediates the auxin-induced expression of the *AIR1* gene by activation of the SCF^{COI1} machinery which then recruits negative regulators of the *AIR1* gene for degradation.

While MeJA was found to induce AIR1 expression, it did not enhance or change *AIR3* expression (Chapter 3). Furthermore, contrary to *AIR1*, the auxin-induced expression of *AIR3* in the *coi1-1* background was not inhibited but even slightly increased. This finding was in agreement with results obtained in induction experiments with auxin in the presence of MeJA and in experiments with MeJA synthesis blockers. So in respect to MeJA regulation *AIR1* and *AIR3* genes are clearly behaving differently. The *AIR1* and *AIR3* genes show almost the same non-induced expression pattern and their auxin-induced expression involve the *ALF4* and *SLR1* genes. These observations indicate that *AIR1* and *AIR3* genes share common regulatory pathways. It can be hypothesized that the auxin-induced expression of the *AIR3* gene is also regulated by a mechanism of ubiquitin-mediated proteolysis of regulators. However, since a mutation in *COI1* does not affect the auxin-induced expression of the *AIR3* gene, different transcription factors might regulate the expression of this gene. It has been found that the transcription factor NAC1, a likely component of the auxin signal transduction pathway for lateral root development, activates the expression of the *AIR3* gene (Xie et al., 2000). NAC1 activity is regulated by SINAT5, a RING-finger protein with ubiquitin E3 ligase activity (Xie et al., 2002). Whether or not NAC1 is a specific regulator of *AIR3* is not known.

To further study the regulation of expression of the *AIR1* and *AIR3* genes, their 5'-flanking sequences were analyzed (Chapter 4). Comparison of *AIR1A* and *AIR1B* 5'-flanking sequences showed that they possessed three highly homologous regions (Neuteboom, 2000). By using information from the *Arabidopsis* genome sequence database, the promoter analysis of the *AIR1A* and *AIR1B* genes was extended to previously unknown 5'-flanking sequences. Two additional highly homologous regions were found. *AIR1B* promoter-deletion::*GUS* expression analysis led to the identification of the region conferring a basal auxin- and MeJA-responsiveness on the promoter, the regions conferring enhanced expression levels to the reporter gene, and a region containing a weak repressor element. Consensus sequences of known auxin- or MeJA-responsive elements were not found. The 5'-flanking sequences of the *AIR3* gene were also analyzed (Chapter 4). The shortest *AIR3* 5'-flanking sequence (1.3 kb) conferring the characteristic *AIR3* expression pattern was identified. Although the *AIR1* and *AIR3* gene expression pattern is almost identical, no similarities were found in the 5'-flanking sequences of these genes.

Information from the *Arabidopsis* genome sequence was used to find the position of *AIR1A, AIR1B* and *AIR3* genes in the *Arabidopsis* genome (Chapter 4). *AIR1A* and *AIR1B* are located in the long arm of chromosome 4 and they are part of a cluster of nine genes encoding putative membrane proteins containing the characteristic CLCT amino sequence at the C-terminal part. In this “CLCT cluster”, two subfamilies of genes were distinguished, one of six members with a proline-rich stretch between the signal peptide and the C-terminal part and one of three members lacking the proline-rich region. The first subfamily corresponds to *pEARLI1* and *pEARLI1-like* genes, a group of early aluminium-induced genes from *Arabidopsis* whose function is unknown (Richards and Gardner, 1995). The other subfamily comprises *AIR1A, AIR1B* and*AIR1-like* gene, a newly identified *AIR1* homologue. These three genes lack the proline-rich region. FASTA analysis indicated that *AIR1A, AIR1B* and *AIR1A-like* are most likely the only genes from *Arabidopsis* encoding CLCT proteins lacking the proline- or glycine-rich region, but still little is known about the function of these types of proteins in plants. *AIR3* is located in the short arm of chromosome 2 at a position where little gene activity is found.

In order to obtain knowledge about the function of *AIR1* and *AIR3* genes the effects of overexpression and inactivation of these genes in *Arabidopsis* were studied. Plants expressing the *AIR1* gene in sense and antisense orientation under the control of a CaMV *35S* promoter showed a normal phenotype (Neuteboom, unpublished results). In this thesis, the effects of *AIR3* overexpression and inactivation were studied (Chapter 5). Plants over expressing *AIR3* mRNA (*35S::AIR3*) showed significantly more lateral root growth than control plants while their primary root length and the lateral root density were not affected. Microscopic analysis indicated that the “long lateral root” phenotype was the result of enhanced meristematic activity in root tip cells. The positive correlation between the expression of the *35S::AIR3* transgene (mRNA levels) and the strength of the lateral root phenotype strongly suggested that a high level of the gene product caused enhanced lateral root elongation. A null mutation in *AIR3* did not cause apparent alterations in the plant.

Nitrate at low concentrations stimulates lateral root elongation while at high concentrations it has an inhibitory effect on lateral root growth (Zhang et al., 1999). In the *35S::AIR3* lines the responsiveness of lateral root growth to nitrate was therefore investigated. In the control lines, growth of lateral roots was inhibited at high nitrate levels, whereas in the *35S::AIR3* lines this effect was reversed. These results indicated

that over expression of *AIR3* was overcoming the inhibitory effect of high nitrate concentrations on lateral root growth, suggesting that nitrate could exert its inhibitory effect by down-regulating *AIR3* expression. This assumption was supported by results obtained with the *AIR3::GUS* reporter line where it was found that GUS activity was strongly down-regulated by high nitrate supply. In contrast to *AIR3*, *AIR1* was not subject to down-regulation by a high level of nitrate.

A model for the mode of action of the subtilisin-like protease AIR3 is proposed. AIR3 may process a precursor protein generating a peptide signal which may be perceived by receptors on the surface of the cells in the adjacent lateral root primordium. Perception of this signal may initiate a signaling cascade leading to enhanced meristematic activity within the primordium causing a swift outgrowth but also to a kind of pre-programming of the meristematic activity of the developing lateral root to meet the nutritional conditions the soil.

Conclusion

The main purpose of this thesis was to gain evidence of the role of *AIR1* and *AIR3* genes in lateral root development and to understand how the expression of these genes is regulated. Although the proposed function of the *AIR1* genes in disconnecting plasma membrane-cell wall connections to facilitate lateral root emergence could not be directly proven, we provide genetic evidence of the link between lateral/adventitious root formation and *AIR1* gene expression. It was previously proposed that the subtilisin-like protease encoded by *AIR3* may be involved in degradation of cell wall components, thereby facilitating lateral root emergence. In this study it was found that *AIR3* is part of the nitrate-signaling network regulating lateral root growth. The auxin-induced expression of the *AIR1* and *AIR3* genes is mediated by a secondary signal. This signal may regulate the expression of these genes by ubiquitin-mediated degradation of specific transcriptional regulators. The secondary signal for the *AIR1* gene is likely to be MeJA, but for the *AIR3* gene the nature of this signal is still unknown.

The results presented in this thesis contribute to a better understanding of the process of lateral root development in which not only the pericycle but also the overlaying cell tissues play a key role. We showed that *AIR1A, AIR1B* and *AIR3*, three

genes specifically expressed in the outer cell layers of the parental root at sites of lateral root formation, are part of a complex network of hormonal and nutritional signals regulating lateral root development.