Chapter 7
The Interplay Between Auxin and the Cell Cycle During Plant Development

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Abstract The essential role of auxin for cell proliferation in plants is well known. Both auxin signaling and cell cycle regulation have been studied elaborately, but less is known about the connection between these processes. Recent studies report on the first molecular pathways that have been found to directly link auxin levels to the regulation of cell cycle activity. Here, we discuss the general effect of auxin on cell cycle progression and then zoom in on the interplay between auxin and the cell cycle during root development in *Arabidopsis thaliana*. At the root tip, an auxin gradient maintains the correct organization of the ground tissue layers and controls the size of the root apical meristem. During auxin-induced lateral root initiation *LATERAL ORGAN BOUNDARIES-DOMAIN* transcription factors are upregulated and control reactivation of the cell cycle and cell specification, both of which are needed for proper lateral root initiation. Auxin-induced lateral root initiation-like pathways are also involved in cell cycle reactivation during the formation of nematode feeding sites, nitrogen-fixing nodules and callus tissue, pointing to the existence of one common auxin–cell cycle module to initiate new organs in plants.

1 Introduction

Auxin has been known for a long time as an important regulator of cell proliferation and raised auxin levels are generally considered as a prerequisite for cell division competence. Although recently much knowledge has been gained about both auxin

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signaling and the regulation of the plant cell cycle, many questions remain unanswered concerning the connection between both processes. During the last years, several studies, mainly on root development, have elucidated molecular pathways that link cellular auxin levels to regulation of the cell cycle machinery. Here, we first give an overview of the general effects of auxin on the plant cell cycle and then discuss the interaction between auxin and cell cycle activity during plant developmental processes. We focus on post-embryonic root development in *Arabidopsis thaliana* (*Arabidopsis*) and particularly on the reactivation of the cell cycle during auxin-induced lateral root initiation.

2 The Cell Cycle Machinery

A regular mitotic cell cycle consists of four different phases. From the G1 gap phase, cells progress toward the S phase, during which DNA is replicated. After a second gap phase, G2, cells divide into two daughter cells at the M phase. The three phases from G1 to G2 are collectively called the interphase. During the gap phases the cells get ready for the upcoming DNA synthesis or mitosis and repair mechanisms are activated when needed. This makes the G1/S and G2/M boundaries important cell cycle checkpoints, which is also shown by the arrest of cells in either G1 or G2 during stressful conditions (Tardieu and Granier 2000; Van't Hof 1985). The switches between the different phases are controlled by the presence of different CDK–CYCLIN complexes. Despite the conservation of the core cell cycle mechanisms in all eukaryotic organisms, there are major differences in the total number of cell cycle regulators. In plants, many more regulators are involved compared to animal and yeast, with over 80 cell cycle proteins known to date in *Arabidopsis* (Menges et al. 2005; Vandepoele et al. 2002; Van Leene et al. 2010). Before we zoom in on how auxin interferes with cell cycle regulation, we first give a summary of the molecular players of the plant cell cycle (Fig. 7.1). For a more profound overview, however, we refer the readers to the review by Inzé and De Veylder (2006).

CYCLIN-DEPENDENT KINASES (CDKs) are the main players in the regulation of the cell cycle. As stated by their name, these serine/threonine kinases are functionally dependent on CYCLINS with which they form protein complexes. The transcription and degradation of CYCLINS is the main mechanism driving cells from one phase of the cell cycle to the next. Most plant D-type CYCLINS (CYCD) are constitutively expressed during the cell cycle, but mainly function during the transition from G1 to S. A-type CYCLINS (CYCA) are most abundant from S to M phase and the majority of B-type CYCLINS (CYCB) are specific to G2 and M phase (Ito 2000; Menges et al. 2005).

Further regulation of the core cell cycle machinery is established by binding of CDK inhibitory proteins to the CDK–CYCLIN complexes. In plants, these inhibitors are known as ICKs (INTERACTOR/INHIBITOR OF CDK) and until now two classes have been identified, being the KIP-RELATED PROTEINS (KRPs) and the

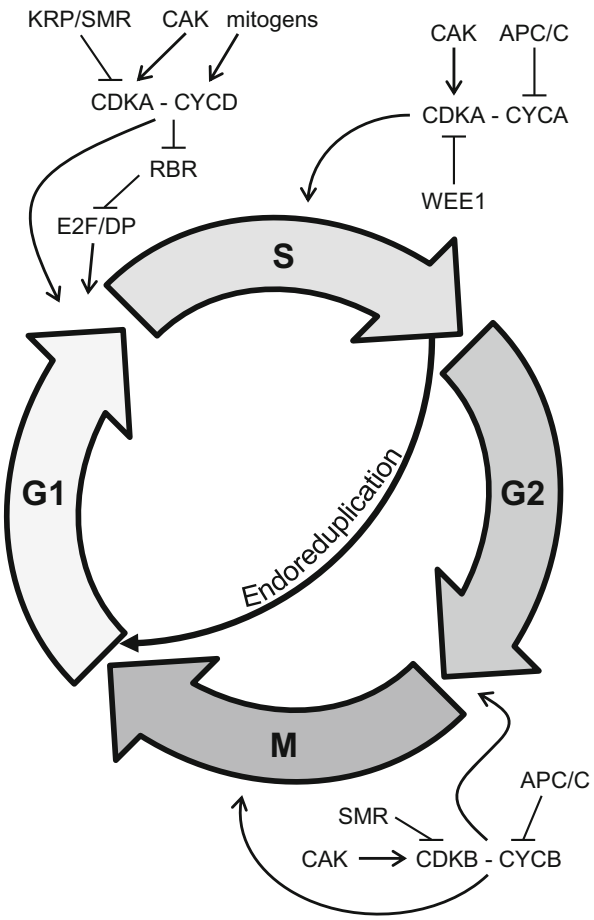

Fig. 7.1 Progression of the plant cell cycle is regulated by the activity of CDK–CYC complexes. The transition from the G1 to S phase is mainly controlled by CDKA–CYCD complexes, which become more abundant in the presence of mitogens due to upregulation of *CYCD* expression. CDKA–CYCD activity inhibits RBR through phosphorylation. Subsequently, E2F/DP activity is released from the inhibition by RBR, and S-phase specific genes are induced. During the S phase, CDKA–CYCA complexes are most important. At the G2/M transition, CDKAs are replaced by CDKBs, which mainly function in collaboration with B-type CYCLINS. For CDKs to be active, they need to be phosphorylated by CAKs, while binding by KRPs or SMRs inactivates them. During S and G2 the activity of CDKAs can also be inhibited by the WEE1 kinase. The APC/C complex is involved in the phase-specific degradation of CYCA and CYCB

SIAMESE (RELATED) (SIM and SMR) proteins (Churchman et al. 2006; Peres et al. 2007). More levels of control arise from protein turnover, which is mainly executed by the SKP1/CULLIN/F-BOX (SCF)-related complex and the ANAPHASE-PROMOTING COMPLEX/CYCLOSOME (APC/C), with the latter specifically functioning during the progression through and exit from mitosis

(Heyman and De Veylder 2012; Vodermaier 2004). CDK activity can also be switched on and off by changes in the phosphorylation status of the CDK itself. CDK-ACTIVATING KINASES (CAKs) mediate the phosphorylation of a conserved threonine residue in the T-loop of the CDKs, which is crucial for CDK activity (Umeda et al. 2005). The WEE1 kinase is responsible for inactivating phosphorylation of the CDKs during S and G2 (Cools et al. 2011; De Schutter et al. 2007; Sorrell et al. 2002; Sun et al. 1999). In most eukaryotes, the activity of WEE1 can be counteracted by the CDC25 phosphatase (Landrieu et al. 2004), but no functional *CDC25* gene has been found in plants.

CDKs are responsible for the phosphorylation of downstream targets, which leads to the breakdown of phase-specific proteins from the previous cell cycle phase and the activation of players in the upcoming phase. At the G1/S transition much of this downstream regulation happens through E2F/DP heterodimer transcription factor activity (Inzé and De Veylder 2006). RETINOBLASTOMA-RELATED (RBR) associates with the E2F/DPs and is a direct target of CDKs. In its unphosphorylated form RBR represses the transcription of E2F/DP target genes. During the G1/S transition, CYCLIN D-dependent CDKs are responsible for RBR phosphorylation, and thus inactivation, thereby enabling the activation of E2F/DP targets.

3 The Interplay Between Auxin and the Cell Cycle

3.1 Auxin Is Needed for Progression Through the Cell Cycle

From studies with tissue cultures, it has been known for many decades that both auxin and cytokinin are important to maintain cell divisions. Furthermore, the ratio between these two plant hormones is a decisive factor to discriminate between root and shoot development from callus tissue and to decide between cell growth and differentiation on the one hand and cell division on the other hand (Murashige and Skoog 1962; Tao and Verbelen 1996). Much of the early work on the effect of auxin and cytokinin on cell cycle progression has been carried out on a diverse set of samples from several plant species such as tobacco, pea and *Arabidopsis*. As a consequence, the apparent discrepancies between the obtained results might have been caused by different characteristics of the used tissues (Bayliss 1985). Also later studies with hormonal treatments *in planta* did not always generate a uniform picture, most likely because of differences in the endogenous hormone levels and transcriptional networks that are active in the samples that were studied (John 2007). As a conclusion from the early experiments on auxin and cytokinin as plant growth regulators, it can be said that both of them are needed to maintain cells in a proliferation competent state. Pinpointing specific cell cycle stages during which these hormones are acting is difficult, since they seem to have a rather general effect on the progression of the cell cycle (see Chap. 12).

3.2 Auxin and Regulation of the Core Cell Cycle Machinery

Many reports demonstrate the influence of auxin treatments on the expression of core cell cycle genes such as *CDKA;1* (Hemerly et al. 1993; John et al. 1993), *CYCB1;1* (Ferreira et al. 1994b), *CYCA2;1* (Burssens et al. 2000), *CYCD3* (Murray et al. 1998; Soni et al. 1995), and *E2Fb* (Magyar et al. 2005). Although auxin treatment increases the expression levels of CDKs (Zhang et al. 1996), it is not able to induce CDK activity. Cotreatment with cytokinin is needed to dephosphorylate and thereby activate the mitotic CDKs (John et al. 1993; Orchard et al. 2005; Zhang et al. 1996, 2005). The effect of auxin on gene regulation is often established through the activity of AUXIN RESPONSE FACTORS (ARFs). These transcription factors bind their target sequences through recognition of auxin response elements (AuxREs, Ulmasov et al. 1995, 1997). For most of the cell cycle genes that have been shown to be transcriptionally regulated by auxin, the involvement of direct binding by ARFs has not been investigated. Many cell cycle genes do however contain AuxREs in their upstream sequence (Table 7.1). The functionality of these elements would need to be tested before a direct link between auxin signaling and the regulation of these genes can be made.

Next to the transcriptional regulation of their gene expression, auxin also targets protein levels of core cell cycle genes. In the case of E2Fb, auxin both increases the expression levels and stabilizes the protein (Magyar et al. 2005). Auxin also binds directly to SKP2A (Jurado et al. 2010), which is an F-box protein that functions in an SCF complex to regulate the ubiquitin-dependent proteolysis of E2Fc and DPb (del Pozo et al. 2002, 2006). The interaction between SKP2A and auxin stimulates both the degradation of SKP2A itself and of its targets (Jurado et al. 2010). The latter is very reminiscent of the TRANSPORT INHIBITOR RESPONSE 1 (TIR1)-AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) auxin signaling pathway (del Pozo and Manzano 2013), in which binding of auxin to the F-box protein TIR1 leads to the degradation of the Aux/IAA proteins and as such activates downstream auxin signaling via ARFs. For more details on the auxin signaling mechanism, we refer readers to the dedicated chapter in this book (see Chap. 6).

SKP2B, the closest homolog of *SKP2A*, is also regulated by auxin. Auxin has been shown to promote histone H3 acetylation in the promoter region of *SKP2B*, which increases its transcription levels (Manzano et al. 2012). KRP1 was reported to be targeted for degradation by SKP2B (Ren et al. 2008), but probably SKP2B also targets positive cell cycle regulators, because it negatively regulates cell proliferation in both apical and lateral root meristems (Manzano et al. 2012).

Modulation of calcium levels is another strategy used by auxin to influence the progression through the cell cycle. Auxin induces a rapid increase of cytosolic Ca^{2+} concentration (Monshausen 2012; Monshausen et al. 2011; Shishova and Lindberg 2010). Through the activity of calcium-dependent protein kinases and phosphatases, these increased Ca^{2+} levels can impact on cell cycle progression. A KRP protein from *Medicago* was shown to have higher activity after calcium-dependent phosphorylation (Pettkó-Szandtner et al. 2006) and regulation of RBR activity by PP2A phosphatases might also depend on Ca^{2+} binding to the PP2As (Dudits et al. 2011).

Table 7.1 Potential binding sites for AUXIN RESPONSE FACTORS in cell cycle genes

Gene name	AGI ID	AuxRE TGTCTC/ GAGACA	AuxRE core TGTC/GACA
CDKA;1	AT3g48750	–	497, 340, 324, 303, 56
CDKB1;1	AT3g54180	–	95
CDKB1;2	AT2g38620	–	–
CDKB2;1	AT1g76540	–	84, 76
CDKB2;2	AT1g20930	–	334, 239, 194, 159, 142, 34
CDKC;1	AT5g10270	–	935, 696, 518, 378, 225, 198
CDKC;2	AT5g64960	–	120
CDKD;1/CAK3	AT1g73690	–	781, 740, 417
CDKD;2/CAK4	AT1g66750	–	873, 353
CDKD;3/CAK2	AT1g18040	–	–
CDKE;1	AT5g63610	–	35
CDKF;1/CAK1	AT4g28980	–	441, 112
CDKG;1	AT5g63370	–	–
CDKG;2	AT1g67580	–	832, 646, 345, 222, 209, 193, 5
CKS1	AT2g27960	161	–
CKS2	AT2g27970	–	75, 19
CYCA1;1	AT1g44110	648, 140	851, 771
CYCA1;2	AT1g77390	–	481, 332, 263, 63, 23, 8
CYCA2;1	AT5g25380	–	782, 767, 728, 550, 377, 340, 292
CYCA2;2	AT5g11300	–	774, 744, 668, 642, 539, 351, 91
CYCA2;3	AT1g15570	–	686, 640, 340, 213, 97, 36
CYCA2;4	AT1g80370	35	794, 560, 516, 362
CYCA3;1	AT5g43080	96	426, 334, 305
CYCA3;2	AT1g47210	–	988, 881, 731, 717, 332, 325, 288, 276, 268, 261
CYCA3;3	AT1g47220	127	743, 681, 573, 471
CYCA3;4	AT1g47230	–	985, 823, 810, 804, 781, 733, 718, 648, 510, 488, 478, 444, 372, 106
CYCB1;1	AT4g37490	859	526, 289, 136
CYCB1;2	AT5g06150	–	355, 237
CYCB1;3	AT3g11520	–	921, 877, 832, 321, 250
CYCB1;4	AT2g26760	981, 759	928, 237
CYCB1;5	AT1g34460	365, 65	819, 733, 441, 347, 315, 305, 179
CYCB2;1	AT2g17620	440	905, 817, 787, 686, 553, 444, 280, 181
CYCB2;2	AT4g35620	–	854, 739, 295, 185
CYCB2;3	AT1g20610	666	856, 763, 471, 228, 78
CYCB2;4	AT1g76310	–	832, 644, 388
CYCB2;5	AT1g20590	–	797, 713, 458, 411, 326, 305, 296
CYCB3;1	AT1g16330	–	182
CYCC1;1	AT5g48640	–	970, 325, 256, 51
CYCC1;2	AT5g48630	–	–
CYCD1;1	AT1g70210	–	–
CYCD2;1	AT2g22490	–	872, 857, 831, 717, 420, 229, 124
CYCD3;1	AT4g34160	–	251, 219, 54
CYCD3;2	AT5g67260	609, 156	362, 263, 166

(continued)

Table 7.1 (continued)

Gene name	AGI ID	AuxRE TGTCTC/ GAGACA	AuxRE core TGTC/GACA
CYCD3;3	AT3g50070	–	997, 958, 870, 509, 366, 208, 154
CYCD4;1	AT5g65420	–	641, 434, 264, 71
CYCD4;2	AT5g10440	–	939, 319, 151
CYCD5;1	AT4g37630	–	659, 610, 514, 260
CYCD6;1	AT4g03270	920	926, 899, 19
CYCD7;1	AT5g02110	–	691, 538, 173, 92
CYCH;1	AT5g27620	–	771, 755, 743, 654, 467, 308, 221, 89, 57
CYCL1	AT2g26430	835, 727, 496	717, 417, 14
DEL1	AT3g48160	–	518, 428, 341
DEL2	AT5g14960	425, 50, 7	563, 488, 334, 169
DEL3	AT3g01330	–	491
DPa	AT5g02470	768	921, 840, 749, 597
DPb	AT5g03415	–	–
E2Fa	AT2g36010	–	692, 604, 584
E2Fb	AT5g22220	478	574, 559, 544, 400
E2Fc	AT1g47870	–	869, 690, 296, 35
KRP1	AT2g23430	330	842, 836, 694, 515, 466, 458, 445, 355, 281, 183
KRP2	AT3g50630	–	970, 909, 876, 666, 620, 589, 539, 184, 67
KRP3	AT5g48820	–	889, 749, 340
KRP4	AT2g32710	–	503, 466, 323, 229
KRP5	AT3g24810	–	988, 925, 847, 519, 99, 76, 61, 16
KRP6	AT3g19150	–	507, 457, 267, 73, 63
KRP7	AT1g49620	407	625, 548, 362, 310, 50
RBR	AT3g12280	–	284, 141, 22
SIM	AT5g04470	–	704, 605, 563
SMR1	AT3g10525	–	393, 354
SMR2	AT1g08180	632	828, 788, 623, 600, 523, 446, 264, 198, 26
SMR3	AT5g02420	10	854, 390, 208, 141, 107
SMR4	AT5g02220	–	687, 375, 318, 12
SMR5	AT1g07500	–	343
SMR6	AT5g40460	–	872, 705, 469
SMR8	AT1g10690	–	846, 787, 691, 365, 242, 167
SMR11	AT2g28330	–	839, 817, 752, 508, 32
WEE1	AT1g02970	–	–

This table gives an overview of the presence of auxin response elements (AuxREs) in the upstream region of core cell cycle regulators. The gene list is based on the work of Menges et al. (2005), Vandepoele et al. (2002), and Van Leene et al. (2010). Since variations of the canonical AuxRE sequence have also been shown to be targeted by ARFs, we present the position of both AuxREs (TGTCTC) and their core sequence (TGTC, Ulmasov et al. 1999; Walcher and Nemhauser 2012; Donner et al. 2009). This overview is limited to 1 kb upstream regions and does not exclude the presence of functional AuxREs in other parts of the promoters. One kb sequences upstream of the transcriptional start site were obtained from PLAZA 2.5 (Van Bel et al. 2012). Shorter sequences were extracted if the upstream gene was situated within less than 1 kb. The position of the elements is indicated as the number of nucleotides upstream of the transcriptional start site. *CKS* CDK-SUBUNIT, *DEL* DP-E2F-LIKE

3.3 Auxin and Endoreduplication

When plant cells start to differentiate, they often switch from the mitotic cell cycle to the endocycle. During the endocycle, or endoreduplication, the cell proceeds from the S phase directly to G1, without passing through G2 and M (Fig. 7.1). This results in a doubling of the DNA content of the nucleus. This process can occur several times, yielding cells with 4C, 8C, 16C, and even higher ploidy levels instead of the usual 2C in *Arabidopsis*. The biological relevance of endoreduplication has long remained elusive, but recent progress indicates that the cell cycle switch to endoreduplication is essential for proper plant development and for responses to the changing environment (De Veylder et al. 2011).

Different effects of auxin on the endocycle have been reported. In tobacco cell cultures depleted of cytokinin, auxin was shown to induce cell elongation that was accompanied by endoreduplication (Valente et al. 1998). High auxin levels have also been correlated with the induction of endoreduplication in differentiating tissues, for example during endosperm development (Sabelli et al. 2007), in apricot and tomato fruits (Bradley and Crane 1955; Chevalier 2007) and in cultured *Petunia* tissue (Liscum and Hangarter 1991). The work of Ishida et al. (2010) on *Arabidopsis* root tips showed however an opposite effect of auxin on cell ploidy levels and on the transition from meristematic cells to differentiating cells in the root apical meristem. Their data indicate that high auxin levels drive cells through the mitotic cell cycle, while the switch to endocycle is only made when less auxin is present. This switch was correlated with a decreased transcription of M phase-specific genes such as *CYCA2;3* and *CYCB1;1* (Ishida et al. 2010). Earlier work from Magyar et al. (2005) showed that auxin stabilizes E2Fb and induces its expression, which in turn stimulates both the G1/S and G2/M transition. As such auxin promotes cell proliferation instead of cell cycle exit and endoreduplication.

This dual effect of high auxin concentrations on endoreduplication clearly shows the importance of the plant developmental context. Depending on the presence of mitosis promoting factors such as cytokinin, auxin stimulates either cell proliferation or endoreduplication. Either way, auxin accumulation enhances the G1/S transition. When concentrations of mitosis-specific factors are high enough, cells will divide. If this is not the case, they will switch to endoreduplication. Since auxin is involved in the regulation of both S and M phase-specific genes, the auxin level itself might be involved in making the difference. This is in accordance with a proposed mechanism for cell cycle progression that depends on an increasing CDK activity from G1/S to G2/M (De Veylder et al. 2011). As such, cells in which auxin is not able to induce high enough CDK activity to cross the G2/M border would start the endocycle. Nevertheless, it is clear that not only the absolute levels of auxin and cytokinin matter for a balance between mitosis and the endocycle, but the ratio between these two plant hormones is as important.

4 An Auxin Gradient at the Root Tip Maintains Pattern and Size of the Apical Meristem

The transcription factors SHORT ROOT (SHR) and SCARECROW (SCR) function as a heterodimer at the root apical stem cell niche to establish the cortex and endodermis ground tissue layers. The continuous production of these layers depends on two successive asymmetric divisions in the cortex-endodermis initial cells and their daughter cells. These divisions are controlled by SHR and SCR and their interaction with RBR. RBR binds to SCR, thereby restricting the functionality of the SHR–SCR heterodimer to induce the formative divisions (Cruz-Ramírez et al. 2012). One of the direct targets of SHR and SCR, amongst other cell cycle genes, is *CYCD6;1* (Sozzani et al. 2010). The CDKB;1–CYCD6;1 complex was shown to phosphorylate RBR, which leads to its inactivation (Cruz-Ramírez et al. 2012). In this way a feedforward loop is established that maintains a bistable circuit in which SHR–SCR activity is either high or low, with high activity inducing asymmetric cell division (Cruz-Ramírez et al. 2012). Auxin can impact on this circuit by its ability to induce *CYCD6;1* expression, although in an SHR–SCR dependent way (Cruz-Ramírez et al. 2012). As such the radial information provided by the cell type-specific expression of SHR and SCR and the longitudinal gradient of auxin at the root tip (Grieneisen et al. 2007, see Chap. 5) together restrict formative divisions to the cortex-endodermis initial cells and their daughter cells. Thereby the correct patterning of the ground tissue is guaranteed. Moreover, Weimer et al. (2012) showed that RBR not only inhibits the transcription of cell cycle genes, but also of genes that are needed for asymmetric divisions and cell fate acquisition. Depending on the activity level of CDKA;1, the RBR-controlled inhibition of only the cell cycle genes or of both cell cycle and specification genes is abrogated. In this way, the asymmetric divisions of the cortex-endodermis daughter cells only occur when CDKA;1 activity is high enough (Weimer et al. 2012).

As described earlier, the auxin gradient at the root tip also controls the size of the root apical meristem. High auxin levels inhibit the transition from the mitotic cell cycle to the endocycle and the coupled transition from meristematic to differentiating cells by affecting the transcription of cell cycle genes (Ishida et al. 2010). The PLETHORA (PLT) transcription factors are involved in maintaining the root meristem size (Aida et al. 2004; Galinha et al. 2007), and their activity can be linked to the auxin gradient. PLT1 and PLT2 are also responsible for correct patterning of the root stem cell niche, in parallel with SCR and SHR. Both *PLT* genes are inducible by auxin via an ARF-dependent pathway (Aida et al. 2004). Their expression follows the auxin gradient at the root tip and determines the size of the meristem: high PLT activity close to the quiescent center maintains stem cell identity, medium levels are linked to mitotic activity of the stem cell daughters, and low levels are correlated with cellular differentiation (Aida et al. 2004; Galinha et al. 2007).

5 Auxin Regulates Cell Cycle Reactivation During Lateral Root Initiation

Two important features of lateral root initiation (LRI) have made it a favorable model system to study the interaction between auxin and the cell cycle. First, the inductive effect of auxin treatment on the formation of lateral roots has been known for a long time (Torrey 1950). An increase of endogenous auxin levels results in an overproliferation of lateral roots as well (Boerjan et al. 1995; Celenza et al. 1995; Delarue et al. 1998; King et al. 1995). Second, lateral roots originate from differentiated pericycle cells. The mitotic cell cycle thus needs to be reactivated in these cells to allow the development of new meristematic regions for the production of lateral root primordia. During the last decades many studies have been performed to unravel the tight link between auxin signaling and cell proliferation during the initiation of lateral roots in *Arabidopsis*. After a general introduction to lateral root development, we will give an overview of the interplay between auxin and the cell cycle during LRI.

5.1 Lateral Root Development in Arabidopsis

In *Arabidopsis*, lateral roots originate from those pericycle cells that are located opposite the two xylem strands of the vascular tissue. These cells undergo a series of divisions which lead to the formation of a dome-shaped primordium that emerges through the overlaying tissues (Malamy and Benfey 1997). The first division is asymmetric and is thought to follow respecification of the cells. The primordium becomes an independent lateral root when a new meristem has been established at its tip (Laskowski et al. 1995). When treated with auxin, all xylem pole pericycle cells can be stimulated into LRI, but under normal conditions only a subset of these cells will actually start to proliferate (Himanen et al. 2002). This specification process is preceded by the occurrence of auxin response oscillations in the basal meristem of the main root tip (De Rybel et al. 2010; De Smet et al. 2007; Moreno-Risueno et al. 2010). The basal meristem is the region immediately shootward of the root apical meristem where both cell division and cell elongation occur. The auxin response oscillations take place in the protoxylem strands and induce priming of the neighboring pericycle cells. Hereafter, auxin response maxima are maintained as static spots of expression of the *pDR5::luciferase* auxin response marker (Moreno-Risueno et al. 2010). These spots are referred to as prebranch sites and predict the location where later a lateral root might, but not necessarily will be initiated (Moreno-Risueno et al. 2010; Van Norman et al. 2013). In the prebranch sites, some primed pericycle cells will get specified as lateral root founder cells higher up in the root. Only these specified founder cells will eventually give rise to new lateral root primordia. The moment when the founder cells start to divide is what we refer to as lateral root initiation. It is preceded by increased auxin levels

and the occurrence of an auxin signaling maximum in the lateral root founder cells (Benková et al. 2003; Dubrovsky et al. 2008).

From the above, it is clear that auxin is involved in almost every single step of lateral root development, during the formation of prebranch sites, founder cell specification, and primordium initiation, but also later during development and shaping of the primordium. In what follows we only discuss LRI, which coincides with the reactivation of the cell cycle in the founder cells.

5.2 *Pericycle Cells Must Maintain the Capacity to Divide*

As mentioned before, in *Arabidopsis* lateral roots originate specifically from pericycle cells that are located next to the two xylem poles that are present in the diarch vasculature. These cells thus have to remain capable of proceeding through mitotic cell division cycles, in contrast to most other root cells. The active transcription of *CDKA;1* in all pericycle cells indicates that they still have the competence to divide, also in mature tissues (Hemerly et al. 1993; Martinez et al. 1992).

One of the main morphological differences that were noted in different species is the shorter length of the root cell type that produces lateral roots compared to other root cells. Lloret et al. (1989) measured the length of both xylem and phloem pole-associated pericycle cells in onion, pea, and carrot and found that the cell files from which lateral roots originate always contained the shortest cells. Similar results were obtained in radish and *Arabidopsis* (Beeckman et al. 2001; Dubrovsky et al. 2000; Laskowski et al. 1995). The data from Lloret et al. (1989) indicate that also non-lateral root founder cells still divide after having left the meristematic region of the root tip. As such LRI would rather be regulated by a switch from symmetric to asymmetric divisions than by the reactivation of the mitotic cell cycle (Lloret et al. 1989). This hypothesis is in accordance with the data from Dubrovsky et al. (2000) who have shown the occurrence of symmetric, proliferative divisions in cells in between initiating primordia in *Arabidopsis*. However, when LRI is induced, for example by auxin treatment, in mature parts of the root that are located further away from the root meristem, a reactivation of the cell cycle is needed (Laskowski et al. 1995).

5.3 *A Lateral Root Induction System Synchronizes Auxin-Induced Cell Cycle Reactivation*

A great part of the studies on cell cycle regulation during LRI has been done in lateral root inducing conditions. Since lateral root formation occurs in an acropetal manner, different developmental stages of primordia can be found along the length of the primary root. As such it is hard to pinpoint the cells wherein a specific

developmental stage of LRI is occurring. The small number of cells involved in early lateral root development makes these studies even harder. By inducing the initiation of lateral roots with auxin treatment, it is possible to synchronize the development of lateral root primordia over the entire length of the root. This greatly facilitates the study of specific steps during lateral root formation.

A commonly used method to obtain such synchronization of lateral root development and the associated cell cycle reactivation is the lateral root induction system (LRIS) developed by Himanen et al. (2002). Plants are germinated on the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA) to block the formation of lateral roots. Subsequent treatment with the synthetic auxin 1-naphthalene acetic acid (NAA) triggers the simultaneous development of lateral roots at both strands of xylem pole pericycle cells along the entire main root. This system has been used frequently to study the cell cycle reactivation during auxin-induced LRI (for example by de Almeida Engler et al. 2009; Himanen et al. 2002, 2004; Vanneste et al. 2005) and the main findings will be summarized below.

As discussed before, the initiation of lateral roots in the LRIS differs from non-induced initiation, because the latter does not occur in fully matured pericycle cells. A second difference can be found in the cell cycle phase in which the cells are residing before being recruited for LRI. Blakely and Evans (1979) have shown that in radish seedling roots all pericycle cells arrest in G2 and can be stimulated by auxin to reactivate the cell cycle. An arrest in G2 can also clarify the very fast response to auxin treatment, with divisions already occurring 2 h after the start of the treatment (Blakely and Evans 1979). The experiments of Beeckman et al. (2001) also showed evidence for the occurrence of LRI in cells that are residing in the G2 phase. In the LRIS in *Arabidopsis*, however, LRI starts from G1 cells (Himanen et al. 2002, 2004), while recently Jansen et al. (2013a) demonstrated a G2 start using an adapted LRIS in maize.

5.4 *Auxin-Induced Cell Cycle Activity During Lateral Root Initiation*

During naturally occurring and auxin-induced LRI, many cell cycle genes can be found that show changes in their transcriptional regulation. Most of these changes, such as upregulation of A, B, and D-type *CYCLINS*, *E2Fa*, *DPa*, and *CDKB* as well as downregulation of *KRP1* and *KRP2* (Beeckman et al. 2001; de Almeida Engler et al. 2009; De Veylder et al. 1999; Doerner et al. 1996; Ferreira et al. 1994a; Himanen et al. 2002, 2004; Vanneste et al. 2005), are linked to progression of the cell cycle.

Auxin not only represses the transcription of *KRP2* but also lowers its protein levels (Himanen et al. 2002; Richard et al. 2002; Sanz et al. 2011). KRP2 prevents formative divisions in the pericycle and overexpression reduces the number of lateral roots (Himanen et al. 2002). Correspondingly, its expression can be seen

at sites where no lateral roots are to be formed, such as phloem pole pericycle cells and xylem associated pericycle cells opposite developing primordia (Himanen et al. 2002), suggesting a role in the strict bilateral origin of lateral roots in *Arabidopsis*. Sanz et al. (2011) reported that KRP2 binds CYCD2;1, thereby probably keeping the CDKA;1–CYCD2;1 complexes inactive. KRP1 is likely to act in a similar way to KRP2 during LRI. It also inhibits auxin-induced LRI, is downregulated by auxin, and interacts with CDKA;1–CYCD2;1 complexes (Himanen et al. 2002; Ren et al. 2008). Repression of KRP activity and induction of positive cell cycle regulators by auxin can thus account for a reactivation of the cell cycle.

It has however become clear that an induction of cell proliferation is not enough to stimulate lateral root development. Overexpression of the G1/S regulators *CYCD3;1* or *E2Fa/DPa* or of the mitotic *CYCB1;1* induces divisions in the pericycle, but these are symmetric and do not lead to lateral root primordium establishment (De Smet et al. 2010; Doerner et al. 1996; Vanneste et al. 2005). Auxin signaling is thus responsible for cell specification next to reactivation of the cell cycle. Both processes were shown to act through the SOLITARY ROOT (SLR)/IAA14-ARF7-ARF19 auxin signaling module (Fukaki et al. 2002, 2005, 2006; Okushima et al. 2005; Vanneste et al. 2005; Wilmoth et al. 2005).

5.5 *Auxin-Induced Cell Specification During Lateral Root Initiation*

Specification of cells is often correlated with asymmetric cell divisions (De Smet and Beeckman 2011). This is also the case during LRI, in which the first division of the founder cells is preceded by a nuclear migration that leads to the asymmetric nature of the division. Evidence has been found that auxin-dependent expression of the *LATERAL ORGAN BOUNDARIES-DOMAIN/ASYMMETRIC LEAVES2-LIKE* (*LBD/ASL*) gene family is involved in establishing the polarity of these divisions.

Five members of the *LBD/ASL* family have been shown to be transcriptionally regulated by ARF7/19 and to be expressed during lateral root development, being *LBD16/ASL18*, *LBD17/ASL15*, *LBD18/ASL20*, *LBD29/ASL16*, and *LBD33/ASL24* (hereafter referred to as *LBD16*, *LBD17*, *LBD18*, *LBD29*, and *LBD33*, Goh et al. 2012; Okushima et al. 2005). Okushima et al. (2007) showed that ARF7 and ARF19 induce the expression of *LBD16* and *LBD29* through direct binding to AuxREs in their promoter regions. LBD16 activity in the founder cells was later found to be required for nuclear migration and to establish the asymmetric nature of the first divisions during LRI (Goh et al. 2012). Overexpression of *LBD16*, *LBD18*, or *LBD29*, but not *LBD33*, can induce the formation of lateral roots in the *arf7arf19* lateral rootless mutant background (Goh et al. 2012; Lee et al. 2009; Okushima et al. 2007). Moreover, LBD18 and LBD33 were shown to function as a heterodimer to induce the expression of *E2Fa* in lateral root founder cells by

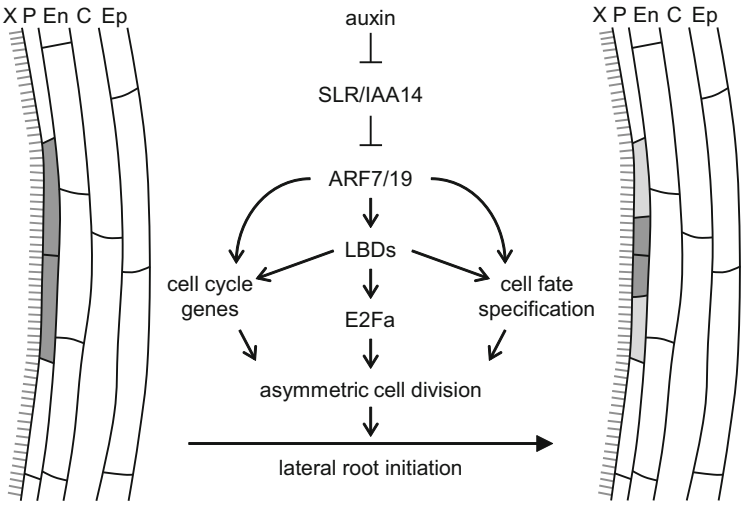

Fig. 7.2 LBD transcription factors regulate cell cycle activity and cell specification during lateral root initiation. During auxin-induced lateral root initiation the cell cycle must be reactivated and the founder cells need to be respecified. Both processes depend on auxin signaling via SLR/IAA14 and ARF7/19. LBD transcription factors are directly targeted by ARF7/19 and subsequently also directly induce *E2Fa* expression. Both ARFs and LBDs probably also target other genes that are involved in cell cycle activity and cell fate specification. As such an asymmetric cell division is established that ensures proper lateral root initiation. *Gray*-colored cells have elevated auxin response levels. *X* xylem, *P* pericycle, *En* endodermis, *C* cortex, *Ep* epidermis

binding directly to its promoter (Berckmans et al. 2011). LBD29 levels have also been linked to changes in the expression of several cell cycle genes (Feng et al. 2012). Altogether, these data indicate that the *LBD* genes play an important role during LRI by linking auxin signaling downstream of ARF7 and ARF19 to cell cycle activity and cell specification (Fig. 7.2).

6 Cell Cycle Reactivation During the Formation of Feeding Sites, Nodules and Callus Has Similarities with Lateral Root Initiation

There are two processes known in plants that depend on the reactivation of the cell cycle in a similar way as is the case for LRI, namely the production of feeding cells by plant-parasitic nematodes and nitrogen-fixing nodule formation in leguminous plants. During both processes the cell cycle is activated in differentiated root cells and in most cases this occurs in the proximity of the xylem poles (Gheysen and Mitchum 2011; Goverse et al. 2000; Grunewald et al. 2009b). Moreover, the development of these organs is linked to an increased auxin response during the

early events (Grunewald et al. 2009a, b; Takanashi et al. 2011; van Noorden et al. 2007), similar to the accumulation of auxin in founder cells before LRI (Benková et al. 2003; Dubrovsky et al. 2008). Concordantly, a large number of proteins were shown to be coregulated during auxin treatment and nodule-inducing *Rhizobium* infection (van Noorden et al. 2007).

Both nematodes and *Rhizobium* bacteria affect auxin distribution, as such generating high auxin concentrations at the sites of infection (Gheysen and Mitchum 2011; Grunewald et al. 2009a, b; Hewezi and Baum 2013; see Chap. 18). This leads, amongst others, to the upregulation of genes involved in cell proliferation. *CDKA;1*, *CDKB1;1*, *CYCA2;1*, and *CYCB1;1* are upregulated during nematode infection, at the site where a feeding cell will be established (de Almeida Engler et al. 1999; Niebel et al. 1996). In the legume *Medicago*, *CYCA2;2* was shown to be upregulated in the proliferating cells involved in nodule formation (Roudier et al. 2003). *KRP1* and *KRP2*, inhibitors of LRI (see higher, Himanen et al. 2002), also have an inhibitory effect on the formation of feeding sites and nodules (Ren et al. 2008; Vieira et al. 2013). All this points to an important role for auxin during the formation of nodules and nematode feeding sites in plant roots. Since the same cell types are involved as for LRI, it can be assumed that similar pathways are activated to translate the auxin signal into stimulation of the cell cycle machinery. This idea is enforced by the fact that nematode infection is correlated with the production of lateral roots at feeding sites (Goverse et al. 2000).

Another process with high similarity to LRI is the in vitro regeneration of plants through callus formation, which also requires elevated auxin levels (Gordon et al. 2007). Callus induction from both root and shoot explants was shown to follow a lateral-root-development pathway (Sugimoto et al. 2010) and callus from root and hypocotyl explants is initiated from xylem-pole-associated pericycle cells (Atta et al. 2009), which are also responsible for LRI. Moreover, xylem-pole-pericycle-like cells were found to be involved in callus formation from cotyledons and petals (Sugimoto et al. 2010).

Four of the LBD transcription factors functioning during LRI are also required for callus induction (Fan et al. 2012; Xu et al. 2012). In line with the data on LRI, upregulation of *LBD16*, *17*, *18*, and *29* during callus formation was shown to be dependent on auxin signaling via ARF7 and ARF19 (Fan et al. 2012). ABERRANT LATERAL ROOT FORMATION 4 (ALF4) is another protein that has been shown to be essential during LRI as well as callus induction (Celenza et al. 1995; DiDonato et al. 2004; Sugimoto et al. 2010). ALF4 functions independently of auxin signaling and seems to have a general effect on cell proliferation competence in different tissues and plant organs (Celenza et al. 1995; Chupeau et al. 2013; DiDonato et al. 2004; Vanneste et al. 2005).

Clearly, a common auxin-dependent pathway is used for the initiation of lateral roots, the induction of feeding sites and nodules and the formation of callus. The distinction between these processes most probably depends on the combination of elevated auxin levels with other growth regulators. An example of this is the switch from callus proliferation to root regeneration when the auxin-to-cytokinin ratio in the growth medium is increased.

7 Perspectives

From all the studies that have been done to clarify the interplay between auxin and the cell cycle, it is clear that a tight link exists between cellular auxin levels and the ability of cells to maintain a proliferative status. For some of the developmental processes that we have described here, factors bridging these auxin levels with cell cycle regulatory genes have been elucidated. There are of course many more processes in which both auxin signaling and cell cycle activity play prominent roles, for example during embryogenesis, the initiation of shoot-derived organs, leaf development, vasculature and cambium development, and lateral root formation beyond the initiation stage. In the future, we expect more links between auxin and cell cycle regulation to be revealed. It will be interesting to find out if identical pathways are employed in distinct plant organs. This seems possible, since, similar to their function in the root tip, SHR, SCR, RBR, and CYCD6;1 activity have been correlated and linked to progression of the cell cycle during leaf development in *Arabidopsis* (Dhondt et al. 2010). Due to the extensiveness of some of the gene families involved in linking auxin to the cell cycle during root development (for example the *Aux*/*IAA*, *ARF*, *CDK*, *CYCLIN* and *LBD* families), it is also plausible that other members of these families will be found to perform similar functions in other tissues. It is however very well possible that other genes will also emerge as signal transducers between auxin and cell proliferation.

Most of the results described here were generated from research on *Arabidopsis*. Comparisons with other species will tell us more about the evolutionary conservation of these pathways. Studies with a recently developed lateral root induction system for maize (Jansen et al. 2013b) will certainly shed more light on this aspect. The first results point toward conserved mechanisms between mono- and dicotyledonous plants concerning auxin-induced lateral root initiation (Jansen et al. 2013a).

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