Running head: Ubiquitin, epigenetic and auxin in lateral root development

Corresponding author: Juan C. del Pozo

Centro de Biotecnología y Genómica de Plantas (CBGP) INIA-UPM. Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria. Campus de Montegancedo, Pozuelo de Alarcón, 28223 Madrid, Spain.

Tlf: +34 91 3364577  pozo@inia.es

Journal research area: Development and Hormone Action
Auxin and epigenetic regulation of 

*SKP2B*, an F-box that represses lateral root formation

Concepción Manzano¹, Elena Ramirez-Parra¹, Ilda Casimiro², Sofía Otero³, Bénédicte Desvoyes³, Bert De Rybel⁴⁵⁶, Tom Beeckman⁴⁵, Pedro Casero³, Crisanto Gutierrez³ and Carlos del Pozo¹*. 

1. Centro de Biotecnología y Genómica de Plantas (CBGP) INIA-UPM. Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria. Campus de Montegancedo, Pozuelo de Alarcón, 28223 Madrid, Spain.

2. Universidad de Extremadura, Facultad de Ciencias, 06006-Badajoz, Spain.

3. Centro de Biología Molecular “Severo Ochoa”, Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Cantoblanco 28049, Madrid Spain

4. Department of Plant Systems Biology, Flanders Institute for Biotechnology, 9052 Ghent, Belgium

5. Department Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium.

6. Current address: Laboratory for Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

* Corresponding author: pozo@inia.es

www.plantphysiol.org on August 22, 2017 - Published by www.plantphysiol.org
Copyright © 2012 American Society of Plant Biologists. All rights reserved.
Financial source: This research was supported by grants from the Spanish Government (BIO2008-00639, BIO2011-28184-C02-01 and CDS2007-0057 to JCP and BFU2009-9783 and CSD2007-000057-B to CG), from the CAM Research Council (S-GEN-0191-2006) to JCP, and from an institutional grant from Fundación Ramón Areces to Centro de Biología Molecular. CM was supported by a predoctoral fellowship from the INIA, SO by a JAE Predoctoral Fellowship from CSIC and BDR by the Special Research Fund of Ghent University (pre-doctoral fellowship).
Abstract

In plants, lateral roots are originated from pericycle founder cells that are specified at regular intervals along the main root. Here, we show that *Arabidopsis thaliana* SKP2B, an F-box protein, negatively regulates cell cycle and lateral root formation as it represses meristematic and founder cell divisions. According with its function, *SKP2B* is expressed in founder cells, lateral root primordia (LRP) and in the root apical meristem (RAM). We identified a novel motif in *SKP2B* promoter that is required for its specific root expression and auxin-dependent induction in the pericycle cells. Next to a transcriptional control by auxin, *SKP2B* expression is regulated by histone H3.1/H3.3 deposition in a CAF-depended manner. *SKP2B* promoter and the 5’ of the transcribed region are enriched in H3.3, which is associated with active chromatin states, over H3.1. Furthermore, the *SKP2B* promoter is also regulated by H3-acetylation in an auxin- and IAA14-dependent manner, reinforcing the idea that epigenetics represents an important regulatory mechanism during lateral root formation.
Introduction

Plants have evolved different root architectures depending on the genotype and on the surrounding environment. Both, the number and position of lateral roots (LR) are major determinants of the root system architecture. Together with root hairs, these lateral organs are responsible for maximizing the surface needed to acquire water and nutrients from the soil. Classical studies (Charlton, 1996) and also more recent work have shown that LR are continuously initiated at a predictable distance above the growing root tip and correlate with the periodic fluctuations in \( DR5 \) expression, a marker that labels the auxin response (De Smet et al., 2007; Moreno-Risueño et al., 2010). Lateral root formation follows an acropetal development, where the lateral root primordia (LRP) are found nearest to the root tip, whereas more mature LR are encountered closer to the root-shoot junction (Fahn, 1974). In Arabidopsis, LR originate from pericycle cells located in front of the xylem poles (Dolan et al., 1993). However, not all of these xylem poles pericycle cells show the same potential to divide since only a few of them, called founder cells, acquire the potential to divide and to form LRP (Casimiro et al., 2003). How these founder cells become specified and differentiated from their neighboring cells? Recent results indicated that the events that determine LR positioning take place in the upper region of the RAM, between the meristem and the elongation zone, in an auxin dependent-manner and involve the Aux/IAA28-dependent auxin-response module (De Smet et al., 2007; De Rybel et al., 2010). This module regulates the expression of \( GATA23 \), a transcription factor involved in founder cell specification (De Rybel et al., 2010). Later, the \( IAA14/SLR \) module regulates the first founder anticlinal cell division as a previous step that triggers the formation of a LRP. The \( slr-1 \) mutation generates a dominant non-degradable IAA14/SLR protein that blocks LR formation (Fukaki et al., 2002). Despite recent advances in identifying the molecular mechanisms that govern the LR position and number, this process is still an intriguing question.

Here, we report on the function of the F-box protein \( SKP2B \) in LR development. \( SKP2B \), which is homologous to the human cell cycle Skp2 (S-Phase Kinase-Associated Protein 2) (del Pozo et al., 2002), regulates the stability of the cyclin-dependent kinase inhibitor \( KRP1 \) (Ren et al., 2008). In this work we show that \( SKP2B \) regulates LR formation by repressing founder cell division. \( SKP2B \) is expressed during the entire LR development and in the root meristem. We have identified a novel motif needed for root specific \( SKP2B \) expression in LRP and founder cells and auxin induction in the pericycle. Using a yeast one-hybrid and ChIP analyses, we found that histone H3.3 binds to \( SKP2B \) promoter. Defects in the histone H3.1/H3.3 deposition alter \( SKP2B \) expression and LR development. Furthermore, we have found that CAF-1, a histone H4/H3.1 chaperone, regulates expression of \( SKP2B \) in the founder cells and LRP. Finally, we demonstrate that acetylation of histone H3 in K9 and K14, two
marks associated with active transcription, occurs in the \textit{SKP2B} promoter and that such modifications are auxin- and IAA14/SLR-dependent.

\textbf{Results}

\textit{SKP2B is expressed in dividing tissues and during early stages of the lateral root initiation}

As \textit{SKP2B} functions in cell cycle, we studied its transcriptional regulation during the cell cycle. \textit{SKP2B} showed two expression peaks that correlate with S and G2/M phases (Supplemental Fig. S1). To analyze its spatio-temporal expression pattern we constructed a transgenic line expressing the GUS reporter under the \textit{SKP2B} promoter (named \textit{SKP2Bp:GUS}). Histochemical GUS staining showed that \textit{SKP2B} was expressed in dividing areas (shoot and root meristems), in the leaf vasculature and in flowers (Fig. 1A-D). In roots, \textit{SKP2B} is expressed in the RAM and in patches along the main root that correlates with LRP in all developmental stages, from stage 0 to VIII (Fig. 1A, 1F-O). Microscopic analyses revealed that \textit{SKP2B} was also expressed in undivided cells close to the root tip that was restricted to pericycle cells at the xylem pole (Fig. 1E-G), likely corresponding to founder cells.

In Arabidopsis, LR formation follows an acropetal sequence of development, with the earliest stages localized close to the root tip. The marker lines \textit{DR5p:GUS} and \textit{GATA23} expression are considered to report the earliest events associated with LR initiation (Benková et al., 2003; Dubrovsky et al., 2008; De Rybel et al., 2010). Comparisons between \textit{DR5p:GUS} and \textit{SKP2Bp:GUS} expression revealed that \textit{SKP2B} was expressed in all morphologically recognizable lateral primordia, including those located between two already developed LRs far away from the RAM (Supplemental Fig. S2A). However, about 20-25\% of the morphologically detected LRP were not stained for the \textit{DR5:GUS} (Supplemental Fig. S2B), indicating the occurrence of fully specified but developmentally arrested LRP having lost the auxin maximum required for further outgrowth.

\textit{SKP2B negatively regulates lateral root formation}

To analyze the role of \textit{SKP2B} in cell division, we crossed the \textit{skp2b} mutant (Ren et al., 2008) with a cell cycle marker, \textit{CYCB1-GUS} (Colon-Carmona et al., 1999). We found that \textit{skp2b} root meristems contain more dividing cells (represented as GUS positive spots, Fig. 2A, B) and bigger root meristem size than wild type (Fig. 2C), indicating higher dividing activity in \textit{skp2b} root meristems. In addition, we also found that the \textit{skp2b} roots were longer than control
roots (Fig. 2D). Next, we analyzed in detail LR formation in the skp2b mutant, finding that skp2b mutants developed more LR (primordia plus emerged LRs) per millimeter than the control (Fig. 2E). When we analyzed the developmental stages of LRP (according to Malamy and Benfey, 1997), we found that 8 days old skp2b roots significantly contained more LRP in stages I and II than control, but we did not observe differences in the number of emerged LR (Fig. 2F). However, when we analyzed 13 days old seedlings, the number of emerged LRs was significantly higher in skp2b than control plants (Fig. 2G). Taken together, these data indicate that SKP2B acts as a repressor of cell division and LR formation.

**SKP2B expression in the root is regulated by auxin**

Auxin signaling plays a central role in the specification of founder cells (De Rybel et al., 2010) and during LRP development (reviewed in Perét et al., 2009). Since SKP2B functions in the LR formation we decided to analyze whether auxin controls the expression of SKP2B in the root. After 3 hours of auxin treatment, SKP2B was initially induced in the pericycle (Fig. 3A), but after 5 or 7 hours, GUS staining was also localized in the surrounding cortex and epidermis, although staining was always stronger in the pericycle layer (Fig. 3A). This data is consistent with the finding that SKP2B expression increases in the pericycle cells after 2 and 6 hours auxin treatment (Parizot et al., 2010). In addition, treatment of SKP2Bp:GUS with 1-N-naphthylphthalamic acid (NPA), which inhibits auxin efflux and blocks LR development, eliminated SKP2B expression in the root, except from the root tip (Fig. 3B). It is possible that NPA impides founder cell specification and LR formation, and consequently SKP2B expression.

To answer this, we grew Arabidopsis seedlings in medium containing 0 or 5 µM of NPA for 7 days. Afterwards, seedlings were transferred to a fresh medium without NPA for 3 extra days and LR were counted only in the root portions that were grown the first 7 days. As shown in Figure 3C, NPA severely compromised, but did not eliminate the pericycle cell competence to further form LRP, suggesting that NPA does not completely block founder cell specification.

Recently, it has been proposed that the auxin response IAA28-module regulates the specification of pericycle cells to become founder cells (De Rybel et al., 2010). The iaa28 mutant can still develop some LRP (Rogg et al., 2001; De Rybel et al., 2010). Corroborating this observation, we found that iaa28/SKP2Bp:GUS plants developed LRP, although significantly fewer than control plants, and all of these LRP expressed SKP2B (Fig. 3D, E). Interestingly, SKP2B was weakly induced by auxin in the iaa28 roots compared to wt roots (Fig. 3F), suggesting a possible role of IAA28 in controlling SKP2B expression. Later, these specified founder cells undergo to an anticlinal cell division to start the development of the LRP. These anticlinal divisions are also controlled by the auxin signaling, involving the activity
of IAA14/SLR. A gain-of-function mutation in IAA14 (slr-1) leads to plants without LR (Fukaki et al., 2002). Histochemical analyses of slr-1/SKP2Bp:GUS showed GUS staining only in the root meristem (Fig. 3G). Auxin treatment of slr-1 did not induce SKP2B expression (Fig. 3G), except for a reproducible expression in few pericycle cells in the differentiation zone (Fig. 3H) that could represent specified founder cells.

Mutations affecting the auxin signaling reduce the number of LR (reviewed in Mockaitis and Estelle, 2008). We crossed SKP2Bp:GUS with auxin signaling mutants (tir1-1, axr1-12 and ibr5-1) reported to develop fewer LR than wild type. We found that auxin-dependent SKP2B induction was impaired in the axr1-12, a strong auxin signaling mutant (Hobbie and Estelle, 1995) (Fig. 4A), while mutations in TIR1 or IBR5 slightly reduced SKP2B induction. Next we studied the number of LR specified in these mutants, finding that tir1-1 and axr1-12 had a fewer number of GUS-stained LRP (Fig. 4B), while ibr5-1 developed similar number than control roots, suggesting that IBR5 activity is needed for the emergence of LR rather than for LR specification, likely due to the function of the IAA28-module is not affected in this ibr5-1 mutant (Strader et al., 2008).

Identification of a novel root-specific expression motif

In order to identify domains responsible for SKP2B expression in founder cells and LRP, we generated different constructs containing deleted versions of the SKP2B promoter. Their expression pattern is summarized in Fig. 5A. A promoter deletion containing 1Kb upstream from the ATG (SKP2B[1Kb]p:GUS) showed similar expression pattern than for SKP2Bp:GUS. However, when we analyzed the expression in the SKP2B[0.5Kb]p:GUS seedlings, the expression was restricted to the founder cells and LRP (Fig. 5B), losing the expression in the aerial part of the plant (data not shown) and in the root meristem (Fig. 5B). When we analyzed the SKP2B[0.34Kb]p:GUS plants, we did not observe any GUS staining, while SKP2B[0.41Kb]p:GUS plants showed a similar expression pattern than SKP2B[0.5Kb]p:GUS plants (Fig. 5A). After auxin treatment, SKP2B[0.5Kb]p:GUS and SKP2B[0.41Kb]p:GUS seedlings showed GUS staining in the pericycle but not in the surrounding cortex nor epidermis (Fig. 5C).

In the process of generating the SKP2B[1Kb]p:GUS lines, we identified a transformation event that did not render GUS staining in the LRP while the root meristem was still stained. After analyzing the insertion by sequencing, we found that this particular SKP2B[1Kb]promoter carried a mutation that replaced the cytosine in position -397 by an
adenine. Next, by directed mutagenesis we generated de novo this mutant construction (SKP2B[1Kb-mut]p:GUS), replacing the same cytosine -397 by adenine. SKP2B[1Kb-mut]p:GUS roots showed GUS staining in the root meristem but not in LRP (Fig. 5D-E), demonstrating the relevance of this residue for its expression in LRP. In addition, this mutation also compromised the SKP2B auxin-dependent induction (Fig. 5F).

With this information, we analyzed the DNA sequence surrounding this cytosine using the PLACE motif search program (http://www.dna.affrc.go.jp/PLACE/index.html) to look for cis-elements. We found a plant motif denominated as “root specific motif” located between nucleotides -387 and -409 from the ATG (Supplemental Fig. S3). We have conducted an in silico analysis to search for promoters that contain at least 1 copy of this root motif, allowing only 1 mismatch in the sequence. We have identified more than 500 genes that contain this motif (Supplemental table I). When comparing these genes with those found in the pericycle cells (De Smet et al., 2008; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6349), we found that about 60% of these genes are expressed in the pericycle cells, while only a percentage of 36.5±3.8 % was obtained when random sampling (3 different random samples of 600 genes each). Moreover, 4% of these genes that contain this motif are induced in response to auxin in the pericycle (Supplemental table I), while random sampling only retrieved 0.15±0.028 %. These data indicate a positive correlation between the presence of this motif and pericycle expression.

**SKP2B promoter is enriched in histone H3.3**

Next, we wanted to get insight in the upstream molecular signaling that controls SKP2B expression in LRP. To do this, we conducted a yeast one-hybrid screening using the SKP2B[0.41Kb] promoter. We chose this promoter region due to the root specific motif identified, fuse to a minimal 35S, was not sufficient to drive SKP2B expression in planta (data not shown). We isolated 10 clones that after re-testing them in a medium containing 5, 10, 15 or 20 mM of 3-AT, only 7 out of them still activated the HIS3 marker (Manzano et al., in preparation). Sequence analysis of these clones revealed that three of them corresponded to the AT5G10980 gene, which encodes for the histone variant H3.3 (Okada et al., 2006). Previously, one-hybrid screenings using other promoters have recovered the three H3 types (Ditzer and Bartels, 2006). However, we did not find other H3 variants in our screening. This data led us to evaluate whether H3.1 could also bind SKP2B[0.41Kb] promoter. We cloned the HISTONE H3.1 and H3.3 in the pGAD424 vector and transformed them into the yeast strain containing the SKP2B[0.41KB]p construct. We observed that yeasts transformed with the H3.1 clone were able
to grow only in the presence of low 3-AT level (5 mM), while those transformed with H3.3 grew up to 20 mM of 3-AT (Supplemental Fig. S4), suggesting that H3.3 has higher affinity for SKP2B promoter than H3.1. This appealing result led us to investigate the H3 status across the SKP2B gene by mapping H3.1 and H3.3 occupancy in DNA extracted from roots. ChIP analyses using plants expressing Myc-tagged H3.1 or H3.3 (Stroud et al., 2012; see methods) followed by PCR amplification of different regions (Fig. 6A) revealed that the SKP2B promoter contained mostly H3.3, while H3.1 was untraceable (Fig. 6B). When the coding region was analyzed, the H3.3 amount increased significantly, but now the H3.1 was detectable, consistent with active transcription of this gene (Fig. 6B). We found that root expressed genes (see methods; Supplemental Fig. S5) also showed H3.3/H3.1 enrichment, but in these cases the levels of H3.1 detected in these promoters were much higher than in SKP2B promoter (Fig. 6B). Since H3.3 is associated with actively transcribed chromatin and SKP2B is highly transcribed in roots upon auxin treatment, we assessed the effect of this hormone on H3.3 deposition. We found that H3.3 deposition did not increase by auxin treatment (Supplemental Fig. S6), suggesting that H3.3 deposition might be more related to specific cell type expression than to auxin response.

HIRA1 chaperone replaces H3.3 for H3.1 in differentiating cells after they exit the cell cycle (Lennox and Cohen, 1988). A HIRA1 homolog was identified in Arabidopsis (Phelps-Durr et al. 2005), but its function is still poorly known and mutations in this gene result in an embryonic lethal phenotype, complicating genetic studies. On the other hand, CAF-1 complex is dedicated to the replication-coupled deposition of H3.1/H4 dimers (Polo and Almouzni, 2006) and viable mutants in Arabidopsis for CAF-1 complex subunits, fas1 and fas2, have been described (Kaya et al., 2001; Serrano-Cartagena et al., 1999). Thus, using this mutants, we decided to study whether alterations in H3.1 deposition influences on SKP2B expression. Histochemical analyses of fas1-4/SKP2Bp:GUS or fas2-1/SKP2Bp:GUS eliminated SKP2B expression in LRP, but not in the main root meristem nor in the LRP surrounding cortex and epidermis (Fig. 6C and Supplemental Fig. S7). We also found that SKP2B auxin induction was compromised in the fas1-4 mutant (Fig. 6D), suggesting that the correct H3.1 incorporation is needed for both its cell specific expression and auxin induction. Unexpectedly, when we analyzed in fas1-4 the expression of SKP2B[0.5Kb]p, a promoter region that specifically drives the expression in LRP, we found a correct GUS staining in LRP (Fig. 6C) and as well as auxin induction in the vascular tissue (Fig. 6D). These data indicate that maintenance of SKP2B expression in founder cells and LRP relays on SKP2B[0.5Kb]p region, but it is influenced by the CAF-1 function in the proximal upstream region.

Next we wondered whether the lack of SKP2B expression in the LRP in the fas1-4 mutant is a general effect on LRP-expressed genes or it is locus specific. To study this, we
generated fas1-4/GATA23p:GUS plants. GATA23 is only expressed in early LRP (De Rybel et al., 2010) and mutation in FAS1 did not affect its expression in early LRP (Supplemental Fig. S7), suggesting that the regulation of SKP2B expression by CAF-1 activity is locus specific and it might represent a good example of how H3.1/H3.3 deposition regulates gene expression.

Finally, using the SKP2Bp:GUS reporter we studied the LR formation in the fas1-4 mutant. We found that fas1-4/SKP2Bp:GUS developed lower number of LRP and emerged LR per root length than SKP2Bp:GUS plants (Fig. 6F), indicating that FAS1 is needed for LR specification and emergence.

**SKP2B promoter is regulated by auxin-dependent histone acetylation**

In addition to histone H3 exchange, H3 acetylation on promoters plays an important role in regulating gene transcription. We carried out ChIP analyses using an antibody that recognizes H3K9ac and H3K14ac and PCR amplification of different regions of the SKP2B promoter. We found that SKP2B promoter was labeled by H3K9/K14ac (Fig. 7A). Next, we analyzed its H3 acetylation level in response to auxin in both wt and slr-1 roots. Interestingly, we found that auxin significantly promotes acetylation in the SKP2B promoter and to a lesser extend in the coding region, and such acetylation was significantly reduced in the slr-1 background. In this mutant, auxin treatment slightly increased the acetylation level in the SKP2B promoter, but never to the control ones (Fig. 7B). Similarly, we detected that root-expressed promoters of CYCB1;1, GRP, PIN6 and ACT2 also contained acetylated H3 (Fig. 7C). The slr-1 dominant mutation also seems to reduce the H3 acetylation level in the root-expressed promoters, but the reduction was significantly lower than in SKP2Bp. The bigger changes were found in the CYCB1;1 promoter, what was expected since the expression of this locus is induced by auxin in the root (Himanen et al., 2002), and in the PIN6 promoter, in which acetylation level was reduced by auxin treatment (Fig. 7C).

Next, we analyzed the effect of trichostatin A (TSA), a histone deacetylase inhibitor, on SKP2Bp:GUS and on the auxin signaling marker DR5p:GUS. Short TSA treatment (12 hours) led to higher and delocalized GUS staining in the basal meristem and transition zone in SKP2Bp:GUS roots (Fig. 7D). TSA-treated DR5p:GUS seedlings also showed a significantly increased GUS staining in the vasculature of the basal meristem and transition/differentiation zone (Fig. 7D), similar to what was found in auxin treated seedlings. Conversely, we found lower levels of GUS staining in the most basal LRP in TSA-treated roots (Figure 7E). Remarkably, when seedlings were grown for 3 days in the presence of TSA, instead of 12 hours, the root growth was significantly delayed (Supplemental Fig. S8A-B). Moreover, we found that 3 day TSA treatment blocked the auxin-dependent induction of SKP2B:GUS and DR5:GUS reporters (Supplemental Fig. S8C-D). It has been described that TSA treatment was able to
promote LR formation in the slr-1 mutant (Fukaki et al., 2006). When slr-1 was treated with TSA for 3 days, we found SKP2B expression in specific cells in the pericycle (Supplemental Fig. S8E), likely corresponding to founder cells. Remarkably, all these SKP2B expression points appeared only on the root sections grown in the presence of TSA. Taken together our data suggest that H3 acetylation regulates auxin responsiveness in the basal meristem and SKP2B expression in the root.

Discussion

SKP2B is a negative cell cycle regulator in the root system

Both, SKP2A and SKP2B were identified by its homology with the human Skp2, which is a key regulator of cell division (del Pozo et al., 2002). SKP2A is an auxin binding F-box protein that functions as a positive regulator of cell division (Jurado et al., 2008; Jurado et al., 2010). Despite the high homology of both F-box proteins, SKP2B functions as a negative regulator of cell division in the root meristem and in the founder cells.

Here, we show that skp2b mutant develops higher number of LRP in stages I and II than wild type roots, suggesting that SKP2B participates in the first anticlinal division of founder cells, idea that is supported by the SKP2B expression pattern. Based on this, we think that SKP2B might contribute to maintain founder cells undivided until the correct developmental time. However, although statistically significant, the increase in the number of LRP is not stunning. This could be explained by the redundant mechanisms that govern the cell division process, and that deprivation of SKP2B function is partially compensated by the function of other proteins, attenuating the skp2b root phenotype. This partial compensation has been shown for other cell cycle proteins such as Cdt1 (Nishitani et al. 2001) or p27/Kip1 (Carrano et al., 1999; Amador et al., 2007; Müller et al., 1997). In addition, this idea is supported by the fact that the double mutant for SKP2B and RKP1 (KPC1-related RING finger protein), another E3 ligase that collaborates in the KRP1 proteolysis (Ren et al., 2008), develops more LRP in early stages than either singles mutants or wt plants (Supplemental Fig. S9). In view of these results, it is reasonable to think that additional and redundant mechanisms govern founder cells division, and that SKP2B function is just one of them.

This role as negative cell division regulator might conflict with the proposed role of SKP2B in the degradation of the cell division repressor KRP1 (Ren et al., 2008). However, it is possible that SKP2B degrades other targets in addition to KRP1, as has been shown for many E3 ligases including HsSkp2, which targets cell cycle repressors such p27 (Kossatz et al., 2004) and cell cycle activators such as E2F1 (Marti et al., 1999) or cyclin E (Yeh et al., 2001). In
addition, histological analyses of the KRP1p:GUS plants shows that KRP1 is not expressed in roots in normal developmental conditions (Gyung-Tae Kim, personal communication).

**Identification of a novel and specific root motif**

Until now, few root-specific motifs have been described. In this work we have identified a promoter domain and a motif that are needed for specific root expression of SKP2B. An *in silico* search using this motif led us to identify more than 500 genes that contain it in their promoters (Supplemental table I). It is remarkable that more than 60% of these genes are expressed in pericycle, suggesting that this motif might be needed for pericycle expression. A mutation in the cytosine in position -397 of this motif blocked SKP2B expression in LRP and almost its auxin responsiveness. An *in silico* search revealed the existence of an Aux-RE down-stream of this motif (Supplemental Figure S3), suggesting that this cytosine might influence on this Aux-RE. One possibility is that this cytosine is regulated by methylation. However we do not think that methylation is important for SKP2B regulation, since SKP2Bp:GUS plants treated with 5-aza-2'-deoxycytidine, a inhibitor of DNA methylation, did not show differences in GUS staining (data not shown). Recently, the screening of a Ds-element enhancer trap lines in Arabidopsis led to the identification of a root-specific promoter in the At1g73160 gene (Vijaybhaskar et al., 2008), which contains a copy of the here identified LR specific motif. Based on these observations, we can conclude that the motif identified in this work is important to confer expression in LRP as well as to auxin response.

Recent works indicated that the IAA28-auxin response module functions in founder cell specification while IAA14/SLR regulates LRP initiation (Vanneste et al., 2005; De Rybel et al., 2010). Confirming previous results we found that *iaa28* mutant develops fewer LRP than wt, and all of these LRP detected expressed SKP2B. However, auxin responsiveness of SKP2B was severely compromised in this mutant, suggesting that IAA28-module regulates the expression of SKP2B in response to auxin. Interestingly, we found that the dominant *slr-1* mutation completely blocks SKP2B expression in founder cells and LRP. It is possible that, just after founder cell specification, degradation of IAA14/SLR in founder cells is needed to maintain founder cell status and, later, to allow the first anticalinal division. This data suggest that in the LR formation program, SKP2B regulates cell division downstream of IAA28 and IAA14/SLR auxin response modules. Despite SKP2B promoter contains two Aux-RE in the promoter, suggesting a direct regulation by IAA28 and/or IAA14, we do not know whether the regulation is directed or not. Further experiments involving ChIP analyses will answer this question.

Here we have shown that SKP2Bp:GUS labeled all morphologically recognizable LRP. Conversely, GUS staining is not detected in all LRP in DR5p:GUS plants, a widely used marker.
to study LR development for being the earliest reporter associated with this process (Benková et al., 2003; Dubrovsky et al., 2008), suggesting that some of these LRP are arrested (Zolla et al., 2010; this work). Taken together, we think that SKP2Bp:GUS is an excellent and trustworthy maker to study LR development in different conditions or mutant backgrounds. For example, the use of this marker has easily showed that mutations in AXR1 affects to LR specification while a mutation in IBR5 affects more to LR emergence than to specification (Fig 4B).

**Epigenetic regulation of SKP2B**

Here, we present evidences that SKP2B is regulated by novel mechanisms involving histone exchange and auxin-dependent acetylation.

Histone H3.1 is incorporated into nucleosomes in dividing cells during the DNA synthesis while H3.3, a replacement variant that can substitute H3.1, is incorporated into nucleosomes during transcription, and it is generally associated with actively transcribed chromatin both in animals and plants. This exchange provides a fast and a dynamic gene activation mechanism of loci that are normally repressed by histone modifications (Ahmad and Henikoff, 2002; McKittrick et al. 2004; Schwartz and Ahmad, 2005; Ingouff et al., 2010). CAF-1 activity is involved in depositing H3.1/H4 dimers during DNA replication. Our data clearly show that SKP2B promoter and gene body are enriched in H3.3 over H3.1 and that the lack of FAS1 activity affects the root development, reducing the LR density, what can be explained by defects in founder cell specification or by defects in the development of LRP. It is remarkable that mutations in FAS1 or FAS2 genes eliminate the expression of SKP2B in LRP but not in the root meristem nor in the cortex/epidermis surrounding the LRP. It is possible that, in CAF-1 mutants, the impossibility of a correct deposition of H3.1 leads to an incorrect deposition of other histones and epigenetic marks, what might difficult the correct exchange for H3.3 and proper transcriptional activation. In animal cells, the disruption of CAF-1 dependent H3.1 incorporation during replication activates an alternative salvage pathway, in which HIRA deposits H3.3 at replication sites (Ray-Gallet et al., 2011). Opposite to mammals wehere CAF-1 function is essential (Quivy at el., 2001), in Arabidopsis mutants for CAF-1 subunits (FAS1 or FAS2) are fully viable, although they show defects in the root and shoot meristems (Kaya et al., 2001; Serrano-Cartagena et al., 1999). Our results indicate that in Arabidopsis the lack of CAF-1 activity eliminates SKP2B expression from LRP. At first glance, this result might be surprising since higher H3.3 incorporation correlates with increase in gene expression. However, in plants, it is unknown how or what type of H3 are deposited in CAF-1 mutants, but knock-out mutants for CAF-1 subunits are fully viable, suggesting that plants have evolved
alternative pathways to overcame this lack of H3.1 deposition. In addition, Arabidopsis has several isoforms of H3.1 and H3.3 that might have different function *in vivo* or use different chaperones for deposition, offering higher versatility. However, additional analyses of how and what types H3 are deposited in different chaperones mutants will be necessary to understand this and they will be subject of futures works.

The fact that mutations in *FAS1* do not impede the expression of *SKP2B[0.5Kb]p:GUS* in LRP suggests that this promoter region acts as an autonomous LRP expression module that is not directly regulated by CAF-1 function in founder cells and LRP. Conversely, the incorrect deposition of H3.1 in *fas1* might lead to inaccurate H3.3 (or other histones) deposition and/or epigenetic marks, altering the activity of a regulatory element located upstream of the [0.5Kb]*SKP2B* promoter that influences on the *SKP2B* expression in LRP. However, we can not discard the possibility that the *fas1*-4 mutation changes the levels of a gene(s) that regulates *SKP2B* expression. At present, *SKP2B* promoter seems to respond only to auxin signaling, which is mainly regulated by the activity of Aux/IAA and ARF transcription factors (Mockaitis and Estelle, 2008). Analyzing the transcriptome of the *fas1* mutant (Schönrock et al., 2006) we did not find changes in any Aux/IAA or ARF, suggesting that that the lack of *SKP2B* expression in LRP in *fas1* is likely due to an incorrect H3.1/H3.3 deposition in its promoter rather than changes in the levels of *SKP2B* regulatory proteins. This *SKP2B* regulation by CAF-1 seems to be specific since *GATA23*, an early expressed gene in LRP, is properly expressed in *fas1*-4 mutant, making the *SKP2B* expression a good example of how H3.1/H3.3 deposition might control gene expression in a specific cell type. Although speculative at this time, it is possible that H3.1/H3.3 exchange on specific loci would be one of the molecular mechanisms involved in founder cells specification in the basal meristem along with other already proposed (De Reybel et al., 2010; Moreno-Risueño et al., 2010).

Acetylation on H3K9 and K14 significantly correlated to changes in locus expression, linking these marks to gene activation (Markowetz et al., 2010). The fact that *SKP2B* promoter accumulates these marks in response to auxin and TSA treatment alters *SKP2B* expression indicate that a correct H3 acetylation is needed for proper *SKP2B* expression, at least, in the
root. Recently, it has been shown that auxin regulates gene transcription by re-adjustments on chromatin epigenetics. Auxin regulates changes in the acetylation levels of several promoters through SAGA-like complexes (Anzola et al., 2010). TOPESS (TPL), a transcriptional co-repressor, influences the auxin-mediated repression through the function of histone deacetylase complexes (Szemenyei et al., 2008). It has been suggested that TPL or a TPL-like might interact with IAA14/SLR to repress, via deacetylases activities, the ARF7/19-auxin signaling during LR development. Auxin treatment causes a significant increase of the H3K9/K14ac levels preferentially in the promoter of SKP2B, and this acetylation is diminished in the slr-1 mutant. Supporting this role of histone acetylation in auxin response and root development, it has been shown that TSA, a histone deacetylase inhibitor, treatment partially rescues the LR formation defect in slr-1 (Fukaki et al., 2006). We have shown that TSA activated SKP2B expression in founder cells/LRP in the slr-1. Since SKP2B was initially expressed at the position of transference to TSA-containing medium and in the most apical part of the roots, we think that TSA is promoting founder cell specification and division of these cells in the slr-1 roots rather than activating the division of pre-specified founder cells. In addition, we show that a short-time TSA treatment induces the auxin response marker (DR5p:GUS) and SKP2B expression in the founder cell specification zone. Conversely, long-time TSA treatment completely blocks the auxin response of both SKP2Bp:GUS and DR5:GUS, suggesting that acetylation/deacetylation balance is critical for auxin responsiveness. In the light of these results it is tempting to speculate that H3.3 deposition and H3 acetylation in K9/K14 in an auxin- and IAA14/SLR-dependent pathway activate SKP2B expression in roots and likely it is needed for founder cell specification. However, to unravel the molecular mechanisms that regulate SKP2B expression and LR formation via IAA14 will require further experiments.
Material and methods

Plant material and constructs

In this work, we have used the following plant: control or wild type (Columbia ecotype), \textit{tir1-1} (Ruegger et al., 1998), \textit{skp2b} (Ren et al., 2008), \textit{axr1-12} (Hbbie and Estelle, 1995), \textit{slr-1} (Fukaki et al., 2002), \textit{iaa28} (Rogg et al., 2001), \textit{ibr5-1} (Monroe-Augustus et al., 2003), \textit{fas1-4} (Ramirez-Parra et al., 2007) and \textit{fas2-1} (Serrano-Cartagena et al., 1999). These plants were grown under sterile conditions on vertically oriented MS (half MS salts, 1% sucrose and 1% plant-agar, Duchefa) plates at 22 °C with 16 hours light and 8 hour dark. For auxin treatment, plants were grown in vertical MS-plates for 5 days and then transferred to MS-liquid medium with 1 μM of 2,4-D for the indicated times. For TSA treatments, seedlings were grown for 5 days in solid vertical MS-plates and then treated with 10 μM TSA (SIGMA) in liquid MS medium for 12 hours. These seedlings were then subjected of GUS staining.

To generate the transgenic lines that harbor the different construct containing the full or deletions of the \textit{SKP2B} promoter fused to GUS, the promoter regions were amplified by PCR and cloned into the pDONOR221 by BP recombination (Invitrogen). Then these promoters regions were mobilized to the pGWB3 (Nakagawa et al. 2007) by LR recombination. The full length promoter containing 1750 bp upstream of the ATG (\textit{SKP2Bp:GUS}) was used to generate transgenic plant in three different Arabidopsis ecotypes: Columbia (\textit{SKP2Bp:GUS}), Landsberg \textit{erecta} (\textit{SKP2Bp:GUS(Ler)}) and Wassilewskija (\textit{SKP2Bp:GUS(Ws)}) using the floral deep method (Clough and Bent, 1998). Several independent transgenic lines were analyzed for GUS staining, all of them showing the same expression pattern. Three tandem repetition of the root specific motif (from -393 to -409) were fused to the -50 35S minimum promoter (Tucker et al., 2002) and transgenic plants were generated. To generate the point position mutant promoter, we used the Quick Change Multi Site Directed Mutagenesis kit (Stratagene) using the pDONOR221-\textit{SKP2B}[1Kb] as a template. We changed the cytosine in position -397 to adenine to generate later the pDONOR221-\textit{SKP2B}[1Kb-mut C(-397)A]. After mutagenesis, the whole DNA was sequenced to discard undesired mutations and then transferred to pGWB3 vector by LR recombination to generate the \textit{SKP2B}[1Kb-mut C(-397)A]:GUS.

GUS assays

Histochemical GUS staining was performed as described in del Pozo et al. (2006). Pictures were taken using a Leica stereomicroscope MZ9.5 with a DCF280 camera or a Leica MD2000 microscope with a DCF300 camera.
Yeast One hybrid

The promoter region of SKP2B containing 410 bp upstream from the ATG was cloned into the gateway adapted pHISi-1 vector to prepare reporter yeast harboring HIS3. The construct was linearized with XhoI and 1 µg was used for yeast transformation using the strain Y187. The transformation was carried out as described in the Matchmaker protocol (Clontech, PT3529-1; http://www.clontech.com/images/pt/PT3529-1.pdf), using a cDNA library generated from mRNA isolated from auxin treated 5 days old Arabidopsis seedlings (kindly provided by W. Gray). The screening was carried out in a Dropout base medium without leucine and histidine and containing 5 mM of 3AT. Approximately 1.2 million yeast transformants were screened, and 10 positive clones were isolated, which were re-grown in a minimum medium containing 5, 10, and 20 mM of 3-AT. The DNA of these positive clones were PCR-amplified and sequenced. The full length cDNA of HISTONE H3.1 and H3.3 were cloned in the pGAD42 to transform into the yeast strain containing the SKP2B[0.41]p version to test the activation potential of both proteins.

Chromatin immunoprecipitation (ChIP) assays

To determine the H3.3 and H3.1 enrichment on promoter, we used Arabidopsis transgenic plant expressing promoter and coding sequence of H3.1 (HTR13; At5g10390) or H3.3 (HTR5; At4g40040) fused in frame to Myc-tag and expressed under their own promoters (Stroud et al., 2012). For the ChIP assays, we used chromatin isolated roots of 7 day-old plants grown in MS agar plates in a 16-hour light 8-hour dark at 22ºC. The ChIP experiment was carried out as in Stroud et al. (2012). Chromatin was immunoprecipitated either with 10 µg of anti Myc antibody, clone 4A6 (Millipore) or anti IgG (Abcam ab6703) used as a negative control. The different promoter fragments were amplified by PCR and resolved in agarose gels. We also amplified promoter regions of root-expressed genes. We used a near-localized SKP2B gene (At1g77100, PIN6), the cell cycle and auxin up-regulated gene CYCB1;1, (At4g37490), an auxin down-regulated gene (GRP, At4g30450) and the ACTIN2 (ACT2, At3g18780). The H3K9K14 ChIP assays and data analysis were carried out basically as previously described (Ramirez-Parra et al., 2007), using chromatin isolated form roots cell of 7 day old plants. We fixed the roots in presence of 3 mM Sodium Butyrate (Sigma) and immunoprecipitation was carried out with anti-H3ac antibody (Usptate-Millipore #06-599). FastStart DNA Master SYBR Green I (Roche) was used for quantitative real-time PCR. Data correspond to the average of two independent biological experiments and three independent qPCR analyses per experiment. Primer sequences and conditions are available upon request.
Root growth assays and microscopic analysis

Primary root length was determined as described previously by (Lucas et al., 2011). All data are the mean value of at least 50 plants, and these experiments were repeated twice, obtaining similar values in each experiment. Data values were statistically analyzed using the t-Student function. Total number and stages of LRP were counted according to methods used previously (Malamy and Benfey, 1997) and root meristem size was calculated based on the number of meristematic cortex cells (Casamitjana-Martinez et al. 2003).

Supplemental Data

Supplemental Table 1: In silico identification of genes containing the root specific motif.

Supplemental Figure 1: SKP2B is cell cycle regulated.

Supplemental Figure 2: DR5p:GUS is not expressed in all LRP.

Supplemental Figure 3: Identification of a root specific motif.

Supplemental Figure 4: H3.3 activates the SKP2B(0.5Kb) promoter in yeast one hybrid.

Supplemental Figure 5: Sequence of promoter used in the ChIP analyses

Supplemental Figure 6: H3.3 deposition in auxin treated roots

Supplemental Figure 7: SKP2Bp:GUS expression in fas1-4 and fas2-1.

Supplemental Figure 8: TSA inhibits auxin induction of SKP2B and DR5

Supplemental Figure 9: LRP density in rkp1 mutant.

Acknowledgments

We thank Sara Navarro for her technical assistance. We also are in debt to W. Gray for providing the cDNA library. We want to thank to O. Navarro for reading and typing of the manuscript. We are also in debt to M. Estelle for providing the axr1-12 and tir1-1 mutants; B. Bartel for the ibr5-1 and iaa28 mutants; M. Tasaka for the slr-1 mutant. This research was supported by grants from the Spanish Government: BIO2008-00639, BIO2011-28184-C02-01 and CDS2007-0057 to JCP and BFU2009-9783 and CSD2007-000057-B to CG), from the CAM Research Council (S-GEN-0191-2006) to JCP, and from an institutional grant from Fundación Ramón Areces to Centro de Biologia Molecular. CM was supported by a predoctoral fellowship from the INIA, SO by a JAE Predoctoral Fellowship from CSIC and BDR by the Special Research Fund of Ghent University (pre-doctoral fellowship).
References

Ahmad K, Henikoff S (2002) The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. Mol Cell 9: 1191-1200

Amador V, Ge S, Santamaria PG, Guardavaccaro D, Pagano M (2007) APC/C(Cdc20) controls the ubiquitin-mediated degradation of p21 in prometaphase. Mol Cell 27: 462-473

Anzola JM, Sieberer T, Orthbauer M, Butt H, Korbel B, Weinhofer I, Mullner AE, Luschnig C (2010) Putative Arabidopsis transcriptional adaptor protein (PROPORZ1) is required to modulate histone acetylation in response to auxin. Proc Natl Acad Sci U S A 107: 10308-10313

Benkova E, Michniewicz M, Sauer M, Teichmann T, Seifertova D, Jurgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115: 591-602

Brady SM, Orlando DA, Lee JY, Wang JY, Koch J, Dinneny JR, Mace D, Ohler U, Benfey PN (2007) A high-resolution root spatiotemporal map reveals dominant expression patterns. Science 318: 801-806

Carrano AC, Eytan E, Hershko A, Pagano M (1999) SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol 1: 193-199

Casamitjana-Martinez E, Hofhuis HF, Xu J, Liu CM, Heidstra R, Scheres B (2003) Root-specific CLE19 overexpression and the soil2/2 suppressors implicate a CLV-like pathway in the control of Arabidopsis root meristem maintenance. Curr Biol 13: 1435-1441

Casimiro I, Beeckman T, Graham N, Bhalerao R, Zhang H, Casero P, Sandberg G, Bennett MJ (2003) Dissecting Arabidopsis lateral root development. Trends Plant Sci 8: 165-171

Charlton WA (1996) Lateral root initiation. In: Y. Waisel, A. Eshel, and U. Kafkafi (eds.). Plant roots: The hidden half. Marcel Dekker, Inc., New York: 149-173

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735-743

Colon-Carmona A, You R, Haimovitch-Gal T, Doerner P (1999) Technical advance: spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. Plant J 20: 503-508

De Rybel B, Vassileva V, Parizot B, Demeulemaere M, Grunewald W, Audenaert D, Van Campenhout J, Overvoorde P, Jansen L, Vanneste S, Moller B, Wilson M, Holman T, Van Isterdael G, Brunoud G, Vuylsteke M, Vernoux T, De Veylder L, Inze D, Weijers D, Bennett MJ, Beeckman T (2010) A novel aux/IAA28 signaling cascade activates GATA23-dependent specification of lateral root founder cell identity. Curr Biol 20: 1697-1706

De Smet I, Tetsumura T, De Rybel B, Frey NF, Laplaze L, Casimiro I, Swarup R, Naudts M, Vanneste S, Audenaert D, Inze D, Bennett MJ, Beeckman T (2007) Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis. Development 134: 681-690

De Smet I, Vassileva V, De Rybel B, Levesque MP, Grunewald W, Van Damme D, Van Noorden G, Naudts M, Van Isterdael G, De Clercq R, Wang JY, Meuli N, Vanneste S, Friml J, Hilson
P, Jurgens G, Ingram GC, Inze D, Benfey PN, Beeckman T (2008) Receptor-like kinase ACR4 restricts formative cell divisions in the Arabidopsis root. Science 322: 594-597

del Pozo JC, Boniotti MB, Gutierrez C (2002) Arabidopsis E2Fc functions in cell division and is degraded by the ubiquitin-SCF(AtSKP2) pathway in response to light. Plant Cell 14: 3057-3071

del Pozo JC, Diaz-Trivino S, Cisneros N, Gutierrez C (2006) The balance between cell division and endoreplication depends on E2FC-DPB, transcription factors regulated by the ubiquitin-SCFSKP2A pathway in Arabidopsis. Plant Cell 18: 2224-2235

Ditzer A, Bartels D (2006) Identification of a dehydration and ABA-responsive promoter regulon and isolation of corresponding DNA binding proteins for the group 4 LEA gene CpC2 from C. plantagineum. Plant Mol Biol 61: 643-663

Dolan L, Janmaat K, Willemsen V, Linstead P, Roberts K, Scheres B (1993) Cellular organisation of the Arabidopsis thaliana root. Development 119: 71-84

Dubrovsky JG, Sauer M, Napsucialy-Mendivil S, Ivanchenko MG, Friml J, Shishkova S, Celenza J, Benkova E (2008) Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. Proc Natl Acad Sci U S A 105: 8790-8794

Fahn A, . (1974) Plant Anatomy. 2nd Edn., Pergamon Press, New York: 49-64

Fukaki H, Tameda S, Masuda H, Tasaka M (2002) Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of Arabidopsis. Plant J 29: 153-168

Fukaki H, Taniguchi N, Tasaka M (2006) PICKLE is required for SOLITARY-ROOT/IAA14-mediated repression of ARF7 and ARF19 activity during Arabidopsis lateral root initiation. Plant J 48: 380-389

Himanen K, Boucheron E, Vanneste S, de Almeida Engler J, Inze D, Beeckman T (2002) Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell 14: 2339-2351

Hobbie L, Estelle M (1995) The axr4 auxin-resistant mutants of Arabidopsis thaliana define a gene important for root gravitropism and lateral root initiation. Plant J 7: 211-220

Ingouff M, Berger F Histone3 variants in plants. Chromosoma 119: 27-33

Jurado S, Abraham Z, Manzano C, Lopez-Torrejon G, Pacios LF, del Pozo JC (2010) The Arabidopsis cell cycle F-box protein SKP2A binds to auxin. Plant Cell 22: 3891-3904

Jurado S, Diaz-Trivino S, Abraham Z, Manzano C, Gutierrez C, Pozo CD (2008) SKP2A, an F-box protein that regulates cell division, is degraded via the ubiquitin pathway. Plant J

Kaya H, Shibahara KI, Taoka KI, Iwabuchi M, Stillman B, Araki T (2001) FASCIATA genes for chromatin assembly factor-1 in arabidopsis maintain the cellular organization of apical meristems. Cell 104: 131-142

Kossatz U, Dietrich N, Zender L, Buer J, Manns MP, Malek NP (2004) Skp2-dependent degradation of p27kip1 is essential for cell cycle progression. Genes Dev 18: 2602-2607

Lennox RW, Cohen LH (1988) The production of tissue-specific histone complements during development. Biochem Cell Biol 66: 636-649

Lucas M, Swarup R, Paponov IA, Swarup K, Casimiro I, Lake D, Peret B, Zappala S, Mairhofer S, Whitworth M, Wang J, Ljung K, Marchant A, Sandberg G, Holdsworth MJ, Palme K,
Pridmore T, Mooney S, Bennett MJ (2011) Short-Root regulates primary, lateral, and adventitious root development in Arabidopsis. Plant Physiol 155: 384-398

Malamy JE, Benfey PN (1997) Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development 124: 33-44

Markowetz F, Mulder KW, Airoldi EM, Lemischka IR, Troyanskaya OG (2010) Mapping dynamic histone acetylation patterns to gene expression in nanog-depleted murine embryonic stem cells. PLoS Comput Biol 6: e1001034

Marti A, Wirbelauer C, Scheffner M, Krek W (1999) Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. Nat Cell Biol 1: 14-19

McKittrick E, Gañén PR, Ahmad K, Henikoff S (2004) Histone H3.3 is enriched in covalent modifications associated with active chromatin. Proc Natl Acad Sci U S A 101: 1525-1530

Mockaitis K, Estelle M (2008) Auxin receptors and plant development: a new signaling paradigm. Annu Rev Cell Dev Biol 24: 55-80

Monroe-Augustus M, Zolman BK, Bartel B (2003) IBR5, a dual-specificity phosphatase-like protein modulating auxin and abscisic acid responsiveness in Arabidopsis. Plant Cell 15: 2979-2991

Moreno-Risueno MA, Van Norman JM, Moreno A, Zhang J, Ahnert SE, Benfey PN (2010) Oscillating gene expression determines competence for periodic Arabidopsis root branching. Science 329: 1306-1311

Muller D, Bouchard C, Rudolph B, Steiner P, Stuckmann I, Saffrich R, Ansorge W, Huttner W, Eilers M (1997) Cdk2-dependent phosphorylation of p27 facilitates its Myc-induced release from cyclin E/cdk2 complexes. Oncogene 15: 2561-2576

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng 104: 34-41

Nishitani H, Taraviras S, Lygerou Z, Nishimoto T (2001) The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase. J Biol Chem 276: 44905-44911

Okada T, Singh MB, Bhalla PL (2006) Histone H3 variants in male gametic cells of lily and H3 methylation in mature pollen. Plant Mol Biol 62: 503-512

Parizot B, De Rybel B, Beeckman T VisuaLRTC: a new view on lateral root initiation by combining specific transcriptome data sets. Plant Physiol 153: 34-40

Peret B, De Rybel B, Casimiro I, Benkova E, Swarup R, Laplaze L, Beeckman T, Bennett MJ (2009) Arabidopsis lateral root development: an emerging story. Trends Plant Sci 14: 399-408

Phelps-Durr TL, Thomas J, Vahab P, Timmermans MC (2005) Maize rough sheath2 and its Arabidopsis orthologue ASYMMETRIC LEAVES1 interact with HIRA, a predicted histone chaperone, to maintain knox gene silencing and determinacy during organogenesis. Plant Cell 17: 2886-2898

Polo SE, Almouzni G (2006) Chromatin assembly: a basic recipe with various flavours. Curr Opin Genet Dev 16: 104-111
Quivy JP, Grandi P, Almouzni G (2001) Dimerization of the largest subunit of chromatin assembly factor 1: importance in vitro and during Xenopus early development. EMBO J 20: 2015-2027

Ray-Gallet D, Woolfe A, Vassias I, Pellentz C, Lacoste N, Puri A, Schultz DC, Pchelintsev NA, Adams PD, Jansen LE, Almouzni G Dynamics of histone H3 deposition in vivo reveal a nucleosome gap-filling mechanism for H3.3 to maintain chromatin integrity. Mol Cell 44: 928-941

Ramirez-Parra E, Gutierrez C (2007) E2F regulates FASCIATA1, a chromatin assembly gene whose loss switches on the endocycle and activates gene expression by changing the epigenetic status. Plant Physiol 144: 105-120

Ren H, Santner A, del Pozo JC, Murray JA, Estelle M (2008) Degradation of the cyclin-dependent kinase inhibitor KRP1 is regulated by two different ubiquitin E3 ligases. Plant J 53: 705-716

Rogg LE, Bartel B (2001) Auxin signaling: derepression through regulated proteolysis. Dev Cell 1: 595-604

Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J, Estelle M (1998) The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast grr1p. Genes Dev 12: 198-207

Schonrock N, Exner V, Probst A, Gruissem W, Hennig L (2006) Functional genomic analysis of CAF-1 mutants in Arabidopsis thaliana. J Biol Chem 281: 9560-9568

Schwartz BE, Ahmad K (2005) Transcriptional activation triggers deposition and removal of the histone variant H3.3. Genes Dev 19: 804-814

Serrano-Cartagena J, Robles P, Ponce MR, Micol JL (1999) Genetic analysis of leaf form mutants from the Arabidopsis Information Service collection. Mol Gen Genet 261: 725-739

Strader LC, Monroe-Augustus M, Bartel B (2008) The IBR5 phosphatase promotes Arabidopsis auxin responses through a novel mechanism distinct from TIR1-mediated repressor degradation. BMC Plant Biol 8: 41

Stroud H, Otero S, Desvoyes B, Ramirez-Parra E, Jacobsen SE, Gutierrez C (2012) Genome-wide analysis of histone H3.1 and H3.3 variants in Arabidopsis thaliana. Proc Natl Acad Sci U S A 109: 5370-5375

Szemenyei H, Hannon M, Long JA (2008) TOLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science 319: 1384-1386

Tucker ML, Whitelaw CA, Lyssenko NN, Nath P (2002) Functional analysis of regulatory elements in the gene promoter for an abscission-specific cellulase from bean and isolation, expression, and binding affinity of three TGA-type basic leucine zipper transcription factors. Plant Physiol 130: 1487-1496

Vanneste S, De Rybel B, Beemster GT, Ljung K, De Smet I, Van Isterdael G, Naudts M, Iida R, Gruissem W, Tasaka M, Inze D, Fukaki H, Beeckman T (2005) Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in Arabidopsis thaliana. Plant Cell 17: 3035-3050
Vijaybhaskar V, Subbiah V, Kaur J, Vijayakumari P, Siddiqi I (2008) Identification of a root-specific glycosyltransferase from Arabidopsis and characterization of its promoter. J Biosci 33: 185-193

Yeh KH, Kondo T, Zheng J, Tsvetkov LM, Blair J, Zhang H (2001) The F-box protein SKP2 binds to the phosphorylated threonine 380 in cyclin E and regulates ubiquitin-dependent degradation of cyclin E. Biochem Biophys Res Commun 281: 884-890

Zolla G, Heimer YM, Barak S (2010) Mild salinity stimulates a stress-induced morphogenic response in Arabidopsis thaliana roots. J Exp Bot 61: 211-224

Figure legends

Figure 1: SKP2B expression. A) SKP2Bp:GUS seedlings were grown for 12 days and then stained for GUS activity. In the right bottom a magnification of the basal root showing the closest stained patches is presented. Dash circles indicate the cross section analyzed in E and picture in F. B) SKP2B expression in the shoot meristem. C) Cotyledon and vascular tissue. D) Flower buds. E) Cross-section of the closet to the root tip stained spot as shown in A and F. Arrow indicates the pericycle cells stained for GUS activity. F-O) Representative pictures of lateral root formation at different developmental stages (St), from stage 0 to stage VIII taken from a single root. P-Q) Pictures of two different and representative root meristems showing different GUS staining in the basal meristem (brackets). Bars: A-C 0.5 mm; E, 20 µm; F-Q, 0.2 mm. En, stands for Endodermis; C, for Cortex; and Ep, for Epidermis cell layers.

Figure 2: SKP2B represses cell division. A) Representative pictures of GUS stained root meristems of control (CYCB1;1p:CYCB1-GUS) and skp2b (skp2b/CYCB1;1p:CYCB1-GUS) seedlings grown for 8 days in MS medium in vertical plates. B) Quantification of CYCB1-GUS spots per meristem in control and skp2b mutant roots. * p < 0.0001 by two-sided t test; n = 30. C) Number of meristematic cortex cells in control and skp2b root meristems. * p < 0.0001 by two-sided t test; n ≥ 12. D) Root length of control and skp2b roots (mm: millimeter). E) Number of lateral root primordial (LRP) plus emerged LR per mm. F) Number of lateral root primordia in different stages per millimeter of main root. *, p < 0.0001 by two-sided t test; n = 12. G) Number of emerged lateral roots in control and skp2b plants grown for 13 days in MS medium. The emerged LRs were counted only in the portion of the root formed during the first 8 days (bracket). *, p < 0.00001 by two-sided t test; n ≥ 35. In all cases, error bars represent means ± standard error of the mean (SEM).
**Figure 3: Auxin regulates SKP2B expression.** A) Histochemical GUS staining of 5 days old SKP2Bp:GUS seedlings treated with 1µM of 2,4-D for 0, 3, 5 or 7 hours. Lower panels show higher magnifications of the root meristem and elongation zone. Bars correspond to 0,5 mm (upper panels) and 0,1 mm (lower panels). B) Histochemical GUS staining of 5 days old SKP2Bp:GUS seedlings grown with or without 10 µM of NPA. Bar corresponds to 0,2 mm. Arrows point to GUS stained LRP. C) Number of emerged lateral root in plants grown in medium with or without 5 µM of NPA for 7 days and then 3 extra days in MS. The emerged LRs were counted only in the portion of the root formed during the first 7 days. *, p < 0.00001 by two-sided t test; n ≥ 40. D) Histochemical GUS staining of root meristems and PLRIS of SKP2Bp:GUS(Ws) (Ws:Wassilewskija ecotype) and iaa28/SKP2Bp:GUS(Ws). Bars represent 0,2 mm. E) Total number of GUS stained spots in SKP2Bp:GUS(Ws) and iaa28/SKP2Bp:GUS(Ws). F) Representative pictures of SKP2Bp:GUS(Ws) and iaa28/SKP2Bp:GUS(Ws) roots of seedlings grown 5 days in MS medium and 1 day in 1 µM of 2,4-D in MS medium. Lower panels show higher magnifications of the root tips. Bars represent 0,5 mm. G) Representative picture of the more basal region of slr-1/SKP2Bp:GUS roots stained for GUS activity after treating them with 0 or 1 µM of 2,4-D for 2 days. The arrows point to stained putative LRP. Bars correspond to 0,2 mm. H) Higher magnification of the GUS stained spot in the slr-1/SKP2Bp:GUS treated with 2,4-D. Bars correspond to 0,05 mm.

**Figure 4: Auxin signaling is needed for SKP2B expression.** A) Histochemical GUS staining of 5 days old SKP2Bp:GUS, tir1-1/SKP2Bp:GUS, axr1-12/SKP2Bp:GUS or ibr5-1/SKP2Bp:GUS roots. Seedlings were grown for 5 days in MS medium and then transferred to a fresh medium containing 0 or 1 µM of 2,4-D for 7 hours. Representative picture of the more basal region of the roots were taken. Bars correspond to 0,5 mm. B) Quantification of the number of LRIP stained for GUS activity in the different mutants described above grown for 6 days in MS medium. Error bars represent means ± standard error of the mean (SEM). * p < 0,00001 by two-sided t test; n ≥ 40.

**Figure 5: Dissection of SKP2B root expression.** A) Representation of the different promoter regions used to generate transgenic plants that show expression in the root meristem, in the LRP or in both. SKP2B[1Kb-mut C(-397)A] construction bears a mutation in position -397 that replaces the cytosine by and adenine. B) Representative pictures of GUS stained roots of SKP2Bp:GUS and SKP2B[0.5Kb]p:GUS plants showing a LRP (upper panel) or a root meristem (lower panel). Bar represents 0,1 mm. C) Representative pictures of GUS stained roots of SKP2B[0.5Kb]p:GUS plants treated with auxin showing staining in the pericycle cells,
close to the root tip (left panel) or in the differentiation zone (right panel). Bar represents 0.1 mm. D) Representative pictures of GUS stained roots of SKP2B[1Kb]p:GUS and SKP2B[1Kb-mut]p:GUS plants showing a LRP (upper panel) or a root meristem (lower panel). Bar represents 0.2 mm. E) Higher magnification of representative pictures of GUS stained roots of SKP2B[1Kb]p:GUS and SKP2B[1Kb-mut]p:GUS plants showing a LRP. Bars represent 0.05 mm. F) Representative pictures of GUS stained roots of 5 days old SKP2B[1Kb]p:GUS and SKP2B[1Kb-mut]p:GUS plants treated with 1 µM of 2,4-D for 12 hours. Bars represent 0.1 mm.

**Figure 6: SKP2B promoter is regulated by histone H3 deposition.** A) Schematic representation of At1g77000 (SKP2B) and localization of the primers used for the ChIP-PCR. B) ChIP-PCR analysis of different SKP2B promoter fragments and root expressed genes using chromatin extracted from roots of 7 day-old H3.3-MYC or H3.1-MYC Arabidopsis seedlings. C) Histochemical GUS staining of SKP2Bp:GUS, fas1/SKP2Bp:GUS and fas1/SKP2B[0.5Kb]p:GUS roots. Seedlings were grown for 6 days in MS medium. Bar corresponds to 0.1 mm or 0.05 mm (right panels). RM: root meristem; LRP: LRP. D) Histochemical GUS staining of SKP2Bp:GUS, fas1/SKP2Bp:GUS and fas1/SKP2B[0.5Kb]p:GUS roots treated with auxin (1 µM of 2,4-D) for 0, 3, 5 or 7 hours. E) Quantification of the root length (millimeters, mm), LRP (emerged plus non emerged) and emerged LR per mm in 10 day old fas1-4/SKP2Bp:GUS and SKP2Bp:GUS seedlings. Error bars represent means ± standard error of the mean (SEM). * p < 0.005 by two-sided t-test; n ≥ 20.

**Figure 7: Acetylation in SKP2B promoter is regulated by auxin.** A) ChiP assays using chromatin isolated form roots of 7 days old wt plants. Three different regions in the promoter (a-c) and one in the coding (d) were PCR amplified and separated in an agarose gel. As a control, the ChiP assays were carried out using an anti-IgG. B) Relative acetylation levels on SKP2B locus. ChiP assays of 7 day-old wild type (WT) or slr-1 mutant Arabidopsis roots treated with or without auxin (aux) using antibodies specific for diacetylated-H3. As a control, the ChiP assays were carried out using an anti-IgG. Quantitative PCR was used for relative quantification. The data was normalize to the levels in WT. a: p < 0.001; b: p<0.02; c: p<0.05 by two-sided t-test; n = 6. Error bars represent means ± standard error of the mean (SEM). C) Relative acetylation levels on promoters of root expressed genes. The data was normalize to the levels in WT. a: p < 0.001; b: p<0.02; c: p<0.05 by two-sided t-test; n = 6. Error bars represent means ± standard error of the mean (SEM). D-E) Representative pictures of the root meristem of five days old SKP2Bp:GUS and DR5p:GUS treated with or without TSA (5 µM) during 12
hours in liquid MS medium. Arrows indicate the first LRP labeled by GUS staining. Bars correspond to 0.1 mm in D and 0.5 mm in E.
| Name                  | Position from ATG | GUS staining in roots |
|-----------------------|-------------------|-----------------------|
| SKP2Bp:GUS           | -1750  +9         | + + + +               |
| SKP2B[1Kb]p:GUS      | -1000  +9         | + + + +               |
| SKP2B[0.55Kb]p:GUS   | -560   +9         | + + + +               |
| SKP2B[0.5Kb]p:GUS    | -500   +9         | + + + +               |
| SKP2B[0.46Kb]p:GUS   | -464   +9         | + + + +               |
| SKP2B[0.41Kb]p:GUS   | -410   +9         | + + + +               |
| SKP2B[0.34Kb]p:GUS   | -340   +9         | + + + +               |
| SKP2B[1Kb-mut]p:GUS  | -1000  +9         | + + + +               |

A. (A) Table showing the position from ATG and GUS staining in roots for different SKP2B promoters.

B. (B) Images showing the Histochemical GUS analysis of SKP2B promoters.

C. (C) Images showing the Histochemical GUS analysis of SKP2B promoters.

D. (D) Images showing the Histochemical GUS analysis of SKP2B promoters.

E. (E) Images showing the Histochemical GUS analysis of SKP2B promoters.

F. (F) Images showing the Histochemical GUS analysis of SKP2B promoters.
**A**

B A T1g77000

**B**

Input

IgG

Anti Myc

| Gene | wt H3.1 H3.3 |
|------|---------------|
| SKP2B a | |
| SKP2B b | |
| SKP2B c | |
| SKP2B d | |
| PIN6 | |
| GRP | |
| CYCB1;1 | |
| ACT2 | |

**C**

fas1/SKP2Bp:GUS

fas1-4/SKP2Bp:GUS

fas1-4/SKP2B[0.5Kb]p:GUS

**D**

SKP2Bp:GUS

fas1-4/SKP2Bp:GUS

fas1-4/SKP2B[0.5Kb]p:GUS

**E**

Root length (mm)

LRP + emerged LR/mm

Root length (mm)
