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The MADS-box *XAANTAL1* increases proliferation at the Arabidopsis root stem-cell niche and participates in transition to differentiation by regulating cell-cycle components

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• **Background** Morphogenesis depends on the concerted modulation of cell proliferation and differentiation. Such modulation is dynamically adjusted in response to various external and internal signals via complex transcriptional regulatory networks that mediate between such signals and regulation of cell-cycle and cellular responses (proliferation, growth, differentiation). In plants, which are sessile, the proliferation/differentiation balance is plastically adjusted during their life cycle and transcriptional networks are important in this process. MADS-box genes are key developmental regulators in eukaryotes, but their role in cell proliferation and differentiation modulation in plants remains poorly studied.
• **Methods** We characterize the *XAL1* loss-of-function *xal1-2* allele and overexpression lines using quantitative cellular and cytometry analyses to explore its role in cell cycle, proliferation, stem-cell patterning and transition to differentiation. We used quantitative PCR and cellular markers to explore if XAL1 regulates cell-cycle components and *PLETHORA1* (*PLT1*) gene expression, as well as confocal microscopy to analyse stem-cell niche organization.
• **Key Results** We previously showed that *XAANTAL1* (*XAL1*/*AGL12*) is necessary for Arabidopsis root development as a promoter of cell proliferation in the root apical meristem. Here, we demonstrate that XAL1 positively regulates the expression of *PLT1* and important components of the cell cycle: *CYCD3;1*, *CYCA2;3*, *CYCB1;1*, *CDKB1;1* and *CDT1a*. In addition, we show that *xal1-2* mutant plants have a premature transition to differentiation with root hairs appearing closer to the root tip, while endoreplication in these plants is partially compromised. Coincidently, the final size of cortex cells in the mutant is shorter than wild-type cells. Finally, *XAL1* overexpression-lines corroborate that this transcription factor is able to promote cell proliferation at the stem-cell niche.
• **Conclusion** *XAL1* seems to be an important component of the networks that modulate cell proliferation/differentiation transition and stem-cell proliferation during Arabidopsis root development; it also regulates several cell-cycle components.

Key words: MADS-box, *XAANTAL1* (*XAL1*), cell-cycle, cyclins, CDKs, endoreplication, *PLETHORA*, proliferation/differentiation, root development, *Arabidopsis thaliana*.

INTRODUCTION

Development depends on the dynamic spatio-temporal modulation of cell proliferation and differentiation during morphogenesis. When such modulation is perturbed, aberrant morphologies, such as tumours, may emerge (Dick and Rubin, 2013). In plants, tumours are rare, in comparison with in animals, probably due to the existence of plant cell walls (Sablowski and Carnier Dornelas, 2014), and also because plant morphogenesis has evolved to plastically adjust to environmental conditions, while maintaining patterns within functional limits (Lempe *et al.*, 2013). In contrast to animals, which largely terminate development during embryogenesis, plants produce new organs during their whole life cycle. Plant growth and morphogenesis rely on two main meristems that maintain a pool of undifferentiated cells at the shoot (shoot apical meristem, SAM) and the root (root apical meristem, RAM) tips (Sarkar *et al.*, 2007; Sablowski, 2011).

The root of *Arabidopsis thaliana* (Arabidopsis) has become a useful system to address the molecular genetic components of the networks underlying the modulation of cell proliferation and differentiation during development (Moubayidin *et al.*, 2010). Within the root stem-cell niche (SCN), the stem or initial cells surrounding the organizer or quiescent centre (QC)

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eventually give rise to the cells of the differentiated tissues of the root, which from its outermost to inner layers are: epidermis, cortex, endodermis, pericycle and vascular cylinder (van den Berg *et al*., 1997). In addition, columella cells formed in the root apex are differentiated from the initials beneath the QC (Dolan *et al*., 1993). As the initial cells divide, daughter cells are displaced outside the SCN where they form the proliferation domain (PD) of the RAM, in which cells attain maximum division rates (Ivanov and Dubrovsky, 2013). After several division cycles, cells start dividing at lower rates at the transition domain (TD) within the meristem, and then stop dividing and start to enlarge at the elongation zone (EZ). Progressively, at the differentiation zone (DZ), cells acquire their final size and differentiated cellular features that are characteristic of each root tissue layer (Dolan *et al*., 1993; Dello Ioio *et al*., 2007; Ivanov and Dubrovsky, 2013).

Cell proliferation and differentiation are modulated by transcriptional regulatory networks that integrate external and internal signals (Boye and Nordstrom, 2003; Farkas *et al*., 2006; Slavov and Botstein, 2011). MADS-domain transcription factors have been shown to be key regulators of plant development (Alvarez-Buylla *et al*., 2000*a*; Messenguy and Dubois, 2003; Smaczniak *et al*., 2012), but their role in modulating cell proliferation and differentiation has not been fully addressed. In previous studies we showed that *XAANTAL1* (*XAL1*/*AGL12*) and *XAANTAL2* (*XAL2*/*AGL14*) two MADS-box factors, are necessary for normal root development and cell proliferation control (Tapia-Lopez *et al*., 2008; Garay-Arroyo *et al*., 2013). *xal1* mutants have shorter roots than wild-type plants with fewer meristematic cells and longer cell-cycle duration, resulting in a diminished cell production rate. Moreover *xal1* differentiated cells are smaller than in wild-type roots (Tapia-Lopez *et al*., 2008).

Understanding the specific role of MADS-domain transcription factors and the networks in which they participate in the modulation of proliferation/differentiation requires exploring if they regulate cell-cycle progression. The network underlying the cell-cycle is complex and seems also to be involved in regulating the transition of cells to endoreplication cycles during cell differentiation (Vanstraelen *et al*., 2009; Fox and Duronio, 2013; Edgar *et al*., 2014). Cell-cycle progression is regulated by cyclin-dependent kinases (CDKs), which associate with cyclins (CYCs) that confer substrate specificity (Lim and Kaldis, 2013). Different CDK/CYC complexes act throughout the cell cycle. The CDKA/CYCD complexes trigger the G1–S phase transition. After DNA replication during G2, CDKA and CDKB associated with A- and B-type CYCs induce G2/M transition and then, at later stages of the M phase, CYCA and CYCB must be degraded by APC/C complex to exit mitosis (Menges *et al*., 2005; Eloy *et al*., 2011).

When RAM cells exit from the proliferative mitotic cycle to the EZ, plant cells may enter into endoreplicative cycles during which their DNA content and cell size increase as a result of DNA synthesis without mitotic cell division (Kondorosi *et al*., 2000; Inze and De Veylder, 2006; De Veylder *et al*., 2011; Edgar *et al*., 2014). Endoreplication is in part induced by inhibition of the activity of mitotic CDK–CYC activity by Kip-related proteins (KRPs) or SIAMESE (SIM) proteins (Morgan, 1997; Walker *et al*., 2000; Schnittger *et al*., 2002; Verkest *et al*., 2005; Boudolf *et al*., 2009).

Here we show that *XAL1* is necessary to promote the transition to differentiation during root development as meristematic cells in the *xal1-2* mutant prematurely transit to the EZ and next to the DZ but are unable to reach the final size of wild-type cells, probably because they attain a limited number of endoreplication cycles at the TD–EZ. We also found, in accordance with our previous data in which the mutant *xal1* showed a longer cell-cycle (Tapia-Lopez *et al*., 2008), that XAL1 positively regulates *CYCD3;1*, *CYCA2;3*, *CYCB1;1*, *CDKB1;1* and *CHROMATIN LICENSING AND DNA REPLICATION FACTOR 1* (*CDT1a*; Castellano *et al*., 2004) expression, as well as *PLETHORA1* (*PLT1*; Aida *et al*., 2004). *XAL1* overexpression lines show a higher number of meristematic cells. Interestingly, some plants of these lines also showed altered SCN with abnormal cell divisions under and at the QC. Hence, *XAL1* seems to be an important component of a network that underlies the modulation of cell proliferation/differentiation in root development, and is responsible for regulating some components of the mitotic and endoreplicative cycles (Ishida *et al*., 2009). Furthermore, our results suggest that such a network is involved in both SCN maintenance and apical–basal root development zonation (proliferation, elongation and differentiation) in Arabidopsis. This network also involves *PLT1* (Aida *et al*., 2004; Galinha *et al*., 2007; Mahonen *et al*., 2014). Alternatively, *XAL1* could be an important link between the two networks.

MATERIALS AND METHODS

Plant materials and growth conditions

The Arabidopsis plants used are Columbia-0 ecotype, except for the *PLT1*::GUS (Aida *et al*., 2004) reporter line which is in WS background. Seedlings were grown on vertical plates with 0·2× Murashige Skoog (Murashige and Skoog, 1962) salts (MP Biomedicals) and 1 % sucrose, as described by Tapia-Lopez *et al*. (2008).

XAL1 overexpression lines

XAL1 cDNA was amplified from the pSR102 clone (Rounsley *et al*., 1995) with the primers CB5F (5′-CGGATCC TCTATGGCTCGTGGAAAGATTCA-3′) and CB6R (5′-CCG GATCCGCTAGAACTGAAATATTTCAC-3′) that include *Bam*H1 restriction sites. This DNA fragment was transferred to pGEM-T Easy (Promega, Madison, WI, USA) to generate the RT150 plasmid. After sequence confirmation the *Bam*H1 cut fragment was cloned into the pBIN-JIT plasmid. Plants were transformed via *Agrobacterium tumefaciens* by floral deeping and plants resistant to kanamycin were selected.

RNA extraction and RT-qPCR

Plants were grown for 5 d post-germination (dpg) and roots from three independent biological replicates (25 plants each) were collected. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and it was reverse-transcribed using Superscript II (Invitrogen). We amplified *PDF2* (AT1G13320) and *UPL7* (AT1G13320) as housekeeping

control genes (Czechowski *et al*., 2005), and their stability across the compared samples was confirmed using NormFinder (http://moma.dk/normfinder-software; Vandesompele *et al*., 2002). Amplification efficiencies were analysed using real-time PCR Miner (Zhao and Fernald, 2005), and relative expression was calculated as in Perez-Ruiz *et al*. (2015). Primer sequences are included in Supplementary Data Table S1.

Microscopy

GUS-stained roots were cleared with Herr solution (Herr, 1971) and visualized by Nomarsky microscopy in an Olympus BX60. For quantification of *CYCB1;1::GUS*-stained cells, wild-type, *xal1-2* and *XAL1*-OE 5.2.5 plants were grown for 1, 3, 5 and 7 dpg and 15 roots for each genotype were mounted on slides and all the spots visualized along the cortex tissue file were counted and used for further statistical analysis (Hacham *et al*., 2011). Root meristem organization was visualized using an Olympus FV1000 confocal microscope after roots were fixed and stained using the pseudo-Schiff protocol (Napsucialy-Mendivil *et al*., 2014).

Quantitative analysis of cellular parameters

For quantitative cellular analysis, roots were mounted in 30 % chloral hydrate and examined with Nomarsky optics. Measurements were performed according to Ivanov and Dubrovsky (1997) and Tapia-Lopez *et al*. (2008). Cell size profiles along the apical–basal axis of the root were obtained by cell size measurements along the cortex file of the root from QC to the adjacent cell of the first primordial hair cell.

Flow cytometry analysis

Arabidopsis complete roots of 1 and 3 dpg and root tips 1 cm long of 6 and 9 dpg were chopped and then incubated in Galbraith's buffer (Galbraith *et al*., 1983). The extracts were filtered with nylon filters of 30 μM (Millipore, Billerica, MA, USA), and nuclei were stained with propidium iodide for 10 min and treated with RNAase (10 μg mL^{-1}). Finally 10000 events were measured, and DNA histograms were generated with the cytometer FACS Calibur package (Becton Dickinson, Franklin Lakes, NJ, USA).

RESULTS

XAL1 *is a positive transcriptional regulator of cell-cycle components*

The fact that *xal1-2* mutants have fewer root meristem proliferating cells and a longer cell-cycle duration in comparison with wild-type roots (Tapia-Lopez *et al*., 2008) suggest that XAL1 could be a regulator of cell-cycle components. Hence we assayed mRNA levels of several cell-cycle components in *xal1-2* roots in comparison with wild-type (Fig. 1A). We found that cyclin *CYCD3;1*, which participates in the G1–S transition (Menges *et al*., 2006), as well as *CYCA2;3* and *CDKB1;1*, which participate in the G2–M transition (Doerner *et al*., 1996; Menges *et al*., 2003; Li *et al*., 2005; Inze and De Veylder, 2006; Boudolf *et al*., 2009), are significantly down-regulated in *xal1-2* in comparison with wild-type roots (Fig. 1B). However, other cell-cycle components as *HISH4*, *CYCA2;1*, *CDKA* and *KRP2* are not significantly affected at their mRNA accumulation levels in the mutant background, indicating that XAL1 regulation is specific for some of the cell-cycle components (Fig. 1B). CDKB1;1 interacts with CYCB1;1 to perform its activity (Weingartner *et al*., 2004). Hence, it is not surprising that *CYCB1;1::GUS* (Colon-Carmona *et al*., 1999) was also diminished in the *xal1-2* background and up-regulated in the overexpression line (*XAL1*-OE 5.2.5), in comparison with wild-type roots (Fig. 2A; Supplementary Data Fig. S1). We also found that *CDT1a*, an essential component for the pre-replication complex during the S phase (Castellano *et al*., 2004), was down-regulated in *xal1-2* mutant compared with wild-type roots (Fig. 2B).

The transcription factors PLT1 and PLT2 are important for meristem function in a dose–response fashion in response to auxins regulating proliferation and endocycle onset (Aida *et al*., 2004; Galinha *et al*., 2007; Ishida *et al*., 2009; Zhou *et al*., 2011). *XAL1* is also induced by auxin (Tapia-Lopez *et al*., 2008). Therefore, we tested if *PLT1* is altered in *xal1-2* roots. We found that *PLT1::GUS* (Aida *et al*., 2004) is down-regulated in *xal1-2* roots in comparison with the wild-type (Fig. 2C), particularly at the QC and initial cells, indicating that XAL1 is a positive regulator of *PLT1* as well.

XAL1 *overexpression lines have increased cell proliferation in the root SCN*

To corroborate the role of *XAL1* in the establishment of SCN (Tapia-Lopez *et al*., 2008), we generated several *XAL1* overexpression (OE) lines under the 35S promoter. We analysed seven 100 % kanamycin-resistant OE lines and focused on two of them, *XAL1*-OE 5.2.5 and *XAL1*-OE 7.9.1, that showed high *XAL1* mRNA expression levels (Supplementary Data Fig. S2). Quantitative cellular analyses of these lines showed that *XAL1* overexpression increases the meristem size and the number of meristematic cells at 5 dpg (Fig. 3A, B). In contrast, cell production rate and the final length of cortex cells in these overexpressing lines were similar to wild-type roots (Supplementary Data Fig. S3). Interestingly, over 50 % of the plants of both OE lines showed roots with cellular pattern alterations in the SCN with increased cell divisions (Fig. 3C).

XAL1 *participates in modulating the transition to cell differentiation in roots*

To test if *XAL1* is involved in regulation of the cell transition from the RAM to the EZ and DZ, we analysed the distance from the QC at which root hairs, which are clear markers of differentiation (Foreman and Dolan, 2001), first appear in *XAL1* loss- and gain-of-function lines. We found that root hairs appeared at shorter distances from the QC in *xal1-2* (Fig. 4A, B, D), while these appeared farther away from the QC in the *XAL1*-OE 7.9.1 line in comparison with wild-type plants (Fig. 4A, C, D). Interestingly, root hairs in the mutant are longer and in the OE line are shorter than in wild-type roots (Fig. 4A–C).

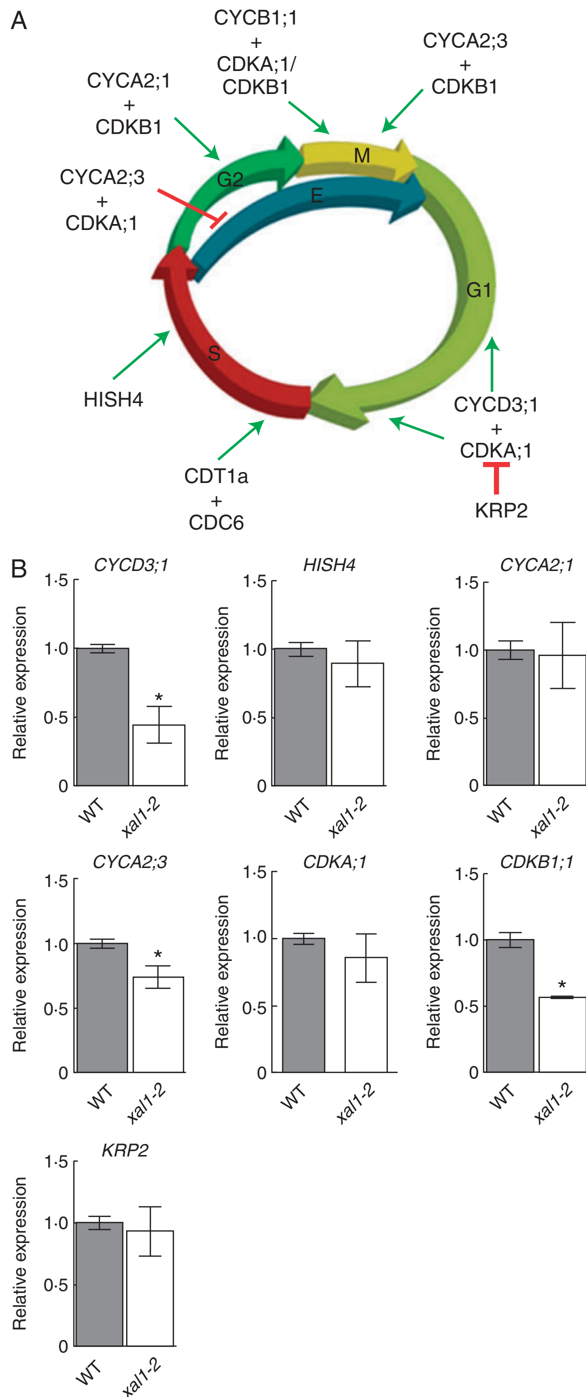

FIG. 1. XAL1 is necessary for *CYCD3;1*, *CYCA2;3* and *CDKB1;1* transcriptional regulation. (A) Schematic representation of the participation of some genes in Arabidopsis cell-cycle transitions. The CYCD3;1/CDKA1 complex triggers G1–S phase by phosphorylation of RB-E2F pathway (not shown) and is essential for cell proliferation. This complex could be inhibited by KRP2 depending on hormonal conditions. CDT1a and CDC6 form the pre-replication complex performing in S phase. During G2 phase, cyclins CYCA2;3, CYCB1;1 and CYCB1;4 are associated with other CDK types (A or B), promoting transition to G2–M and their regulation is important for repressing the endoreplicative cycle. (B) Relative expression levels of some cell-cycle components in *xal1-2* compared with wild-type (WT) roots at 5 dpg, showing that *CYCD3;1*, *CYCA2;3* and *CDKB1;1* are down-regulated in the mutant. Relative expression data are expressed as the mean ± s.d. and statistically significant differences from WT ($*P < 0.05$) were obtained by the Kruskal–Wallis test.

In the transition between the RAM and the EZ, cells exit from the mitotic cycle and go into endoreplicative cycles (Vanstraelen *et al*., 2009). In fact, differentiated cells, such as those with already formed root hairs, show the highest levels of endoreplication in Arabidopsis roots (Abel *et al*., 1995; Guimil and Dunand, 2007; Takahashi *et al*., 2013). Hence, we hypothesized that *xal1-2* roots, in which cells prematurely transit into the EZ and DZ, should also prematurely enter the endoreplication cycle. We used flow cytometry analysis to determine the ploidy levels found in *xal1-2* and the *XAL1*-OE 5.2.5 line compared with wild-type complete roots at 1 and 3 dpg, and root tips (1 cm long) at 6 and 9 dpg to avoid interference caused by lateral roots at these ages (Fig. 4E). Our data showed that 16C ploidy was reduced in *xal1-2* compared with wild-type roots, at 3, 6 and 9 dpg (Fig. 4E). These results suggest that the lack of *XAL1* causes diminished proportions of cells with higher ploidy levels, at the same time that it causes a premature differentiation of cells, with root hairs formed at shorter distances to the QC than in wild-type roots (Fig. 4D). Ploidy levels in the *XAL1*-OE 5.2.5 line are similar to those of wild-type roots (Fig. 4E), although in the former, root hairs appeared farther away from the QC than in wild-type roots (Fig. 4D).

We have previously reported (Tapia-Lopez *et al*., 2008) and corroborated here (Supplementary Data Fig. S2B) that totally differentiated cells in *xal1-2* roots are shorter than wild-type cells, so it is possible that in this mutant, during transition to differentiation, cells are unable to attain normal sizes due to relatively lower ploidy levels (16C) compared with wild-type roots. We measured all the cortex lineage cells from the initial to the first differentiated cell that presented a root hair in *xal1-2* and wild-type roots (Fig. 4F). We found that *xal1-2* cells started to elongate sooner than wild-type cells, but at the equivalent wild-type TD distance from the QC they stopped growing. These data further suggest that *xal1-2* cells transit faster to the elongation and differentiation zones, in comparison with wild-type root cells, but they are unable to endoreplicate at the same rate as in wild-type roots, in correlation with the smaller sizes that they attain in comparison with cells in wild-type roots.

DISCUSSION

We had previously shown that *XAL1* is necessary for normal root growth and development by positively regulating cell proliferation rates and cell elongation, and modulating cell-cycle duration in Arabidopsis roots (Tapia-Lopez *et al*., 2008). Hence, we tested here if *XAL1* regulates cell-cycle regulators.

XAL1 *regulates some cell-cycle components*

We found that expression levels of G1–S factors *CYCD3;1* and *CDT1a* (Menges *et al*., 2006) and G2–M check-point factors *CYCA2;3, CYCB1;1* and *CDKB1;1* (Doerner *et al*., 1996; Menges *et al*., 2003; Li *et al*., 2005; Inze and De Veylder, 2006; Boudolf *et al*., 2009) are significantly diminished in *xal1-2* mutant roots in comparison with wild-type (Figs 1 and 2). Also, we found that overexpression of *XAL1* results in up-regulation of *CYCB1;1::GUS* expression (Fig. 2A; Fig. S1). In agreement with these results, *xal1* mutant alleles have shorter meristems with fewer meristematic cells and the *XAL1*-OE lines

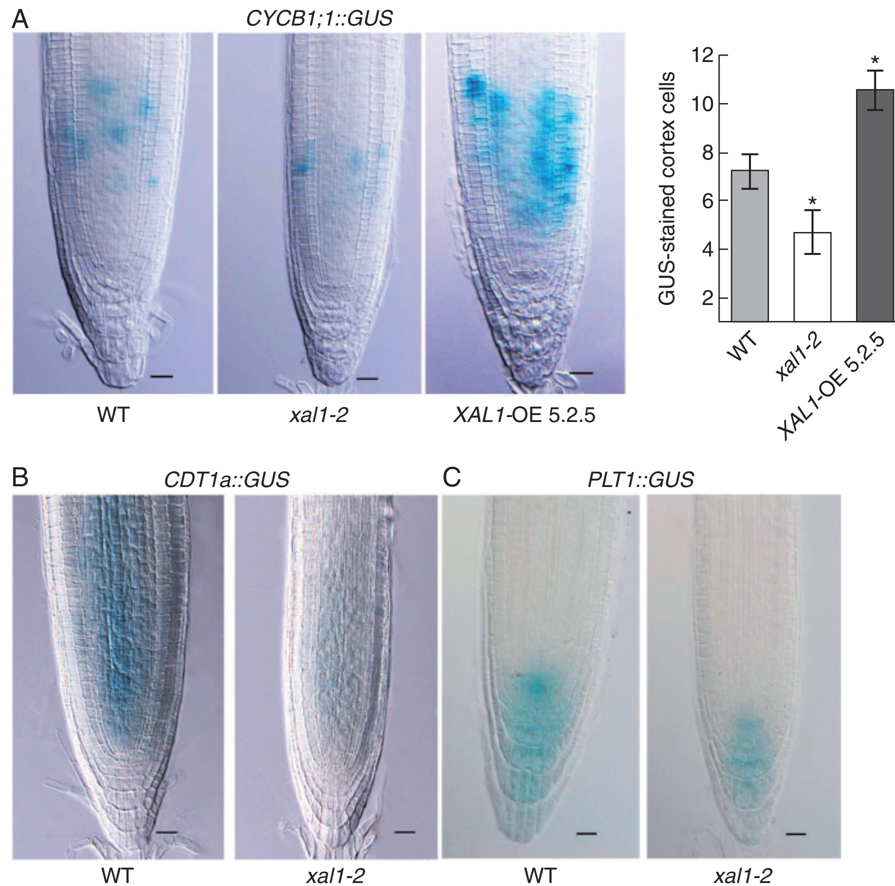

FIG. 2. XAL1 positively regulates *CYCB1*, *CDT1* and *PLT1* in the RAM. (A) Lower and higher levels of *pCYCB1;1::GUS* expression in *xal1-2* roots and *XAL1*-OE 5.2.5 respectively, compared with WT. The number of GUS-stained cortex cells is shown on the right. Data correspond to mean ± s.e. and statistically significant (*) differences from WT ($P < 0{\cdot}05$) were determined with the Kruskal–Wallis test. (B, C) Expression of *pCDT1a::GUS* (B) and *PLT1::GUS* (C) is diminished in *xal1-2* roots compared with WT roots. All plants are 5 dpg, $n = 10$ per line. Scale bars = 20 µm.

have a higher meristematic cell number than wild-type plants (Fig. 3A, B; Tapia-López *et al.*, 2008).

CYCD3;1 forms a complex with CDKA;1 to phosphorylate RBR1, which in turn releases it from E2F transcription factors. This process regulates entry to the cell-cycle and it is required for cell-cycle transitions (Nakagami *et al.*, 1999, 2002; Uemukai *et al.*, 2005; Magyar *et al.*, 2012). The triple mutant *cycd3;1 cycd3;2 cycd3;3* has smaller organs with fewer cells (Dewitte *et al.*, 2007), thus confirming the role of CYCD3 proteins in cell proliferation. Therefore, it is possible that XAL1, a MADS-box transcription factor, promotes cell proliferation by up-regulating *CDKB1;1*, *CYCB1;1*, *CYCA2;3* and *CYCD3;1* at least. Also, lower levels of *CDT1a* in *xal1-2*, a component of the pre-replication complex (Castellano *et al.*, 2004; Sanchez *et al.*, 2012), could explain the longer cell-cycle observed in *xal1* root meristem (Tapia-Lopez *et al.*, 2008).

Down-regulation of *CYCA2;3*, *CYCB1;1* and *CDKB1;1* in *xal1-2* should also favour endocycle entry (Schnittger *et al.*, 2002; Boudolf *et al.*, 2004; Sablowski and Carnier Dornelas, 2014), but as mentioned above, premature enlargement of the meristematic cells does not affect the first rounds of endoreplication. Also we did not find alterations in *KRP2* or *CDKA;1* mRNA expression levels (Fig. 1). These latter components participate in endocycle/mitosis decisions, indicating that at least their transcriptional regulation is independent of XAL1.

Our results suggest that XAL1 regulates both cell proliferation at the meristem and endocycle maintenance during differentiation and it seems that this transcription factor is one of the interconnecting players of the networks underlying these two processes, or they are both regulated by the same network in which XAL1 participates.

MADS-domain factors seem to share some functions among all eukaryotes as suggested by the high conservation of the DNA-binding domain sequence in all lineages of the MADS-domain protein family (Alvarez-Buylla *et al.*, 2000*b*). In animals, MEF-related MADS-domain factors, as is XAL1, have been also identified as critical components of the mechanisms involved in cell proliferation/differentiation decisions in myoblasts by regulating E2F (Naya and Olson, 1999).

XAL1 *mediates the transition to cell differentiation*

We analysed cells transition from the RAM to EZ to the DZ in loss- and gain-of-function lines of *XAL1*. Cells in the *xal1-2* mutant showed premature transition to the elongation and differentiation zones, while this transition was delayed in the

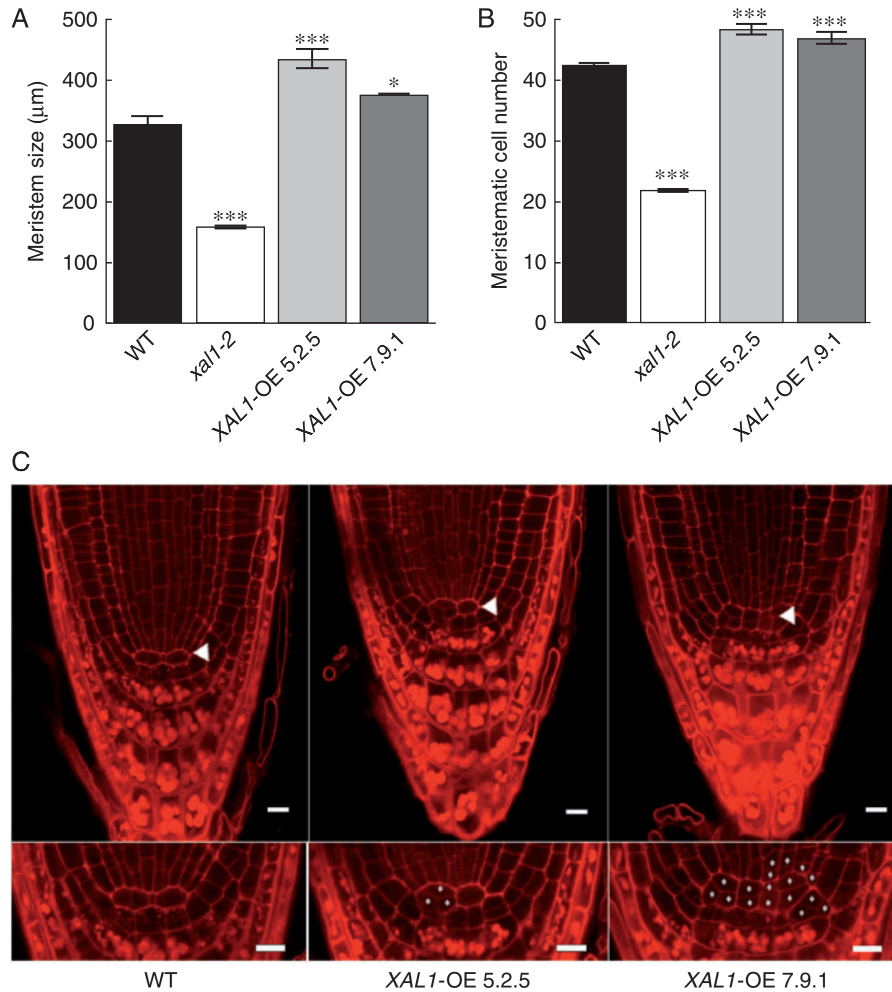

Fig. 3. Overexpression of *XAL1* is sufficient to promote root cell proliferation. (A, B) Meristem size (A) and meristematic cell number (B) are higher in the overexpression lines (*XAL1*-OE 5.2.5 and *XAL1*-OE 7.9.1) while *xal1-2* has both parameters diminished compared with WT roots at 5 dpg. Data correspond to mean ± s.e. Statistical significance (***$P < 0.001$, *$P < 0.05$) was obtained using the Kruskal–Wallis test ($n = 30$). (C) *XAL1*-overexpression increases cell divisions at the SCN. Confocal microscopy showing atypical divisions of the QC (arrowheads) and initial cells (labelled cells in the insets) in 50 % of the *XAL1*-OE 5.2.5 and 7.9.1 plants compared with WT roots. Seedlings of 3 dpg were stained with the pseudo-Schiff technique. $n = 35$ (WT), 20 (*XAL1*-OE 5.2.5) and 32 (*XAL1*-OE 7.9.1) plants. Scale bars = 10 µm.

XAL1-OE lines (Fig. 4), where we found smaller and larger meristems in the *XAL1* loss- and gain-of-function lines, respectively (Fig. 3A, B). Concordantly, we found that in *xal1-2*, root cells start to elongate and differentiate at positions closer to the QC, in comparison with wild-type roots, and we observed the opposite in the *XAL1*-OE lines (Fig. 4). These results imply that *XAL1* participates in the network that underlies the correct timing at which cells transit to a differentiation state.

Some mutants with short root phenotypes that are involved in DNA replication mechanisms show smaller meristems and differentiated cells similar to *xal1*, but with overall diminished ploidy levels and an accumulation of 2–4C cells in comparison with wild-type roots (Breuer *et al*., 2007; Dittmer *et al*., 2007; Kirik *et al*., 2007; Zhou *et al*., 2011). Here we demonstrated premature and delayed transitions to differentiation in the mutant and OE lines of *XAL1*, respectively. Therefore, we expected to find a correlation with larger proportions of cells with higher ploidy levels in the former and lower in the latter, in agreement with studies that have demonstrated that prematurely differentiated cells present higher ploidy levels in their nuclei (Perilli *et al*., 2012). Contrary to this expectation, we found lower proportions of 16C in the *xal1* mutant and no change in ploidy levels in the OE line compared with wild-type roots (Fig. 4E), thus indicating that *XAL1* is necessary for maintaining normal high ploidy levels in the root cells but is not sufficient to alter them. It has been established that there is a correlation between cell size and high ploidy levels (Kondorosi *et al*., 2000; Sugimoto-Shirasu and Roberts, 2003; Inze and De Veylder, 2006). Our results indicate that cells in *xal1-2* start elongating earlier than wild-type cells and as a consequence they differentiate earlier as well, affecting their normal final size (Fig. 4F; Supplementary Data Fig. S3B). Mutants in components of the topoisomerase VI and II are unable to progress into endoreplicative cycles beyond 8C ploidy. Thus, mutants

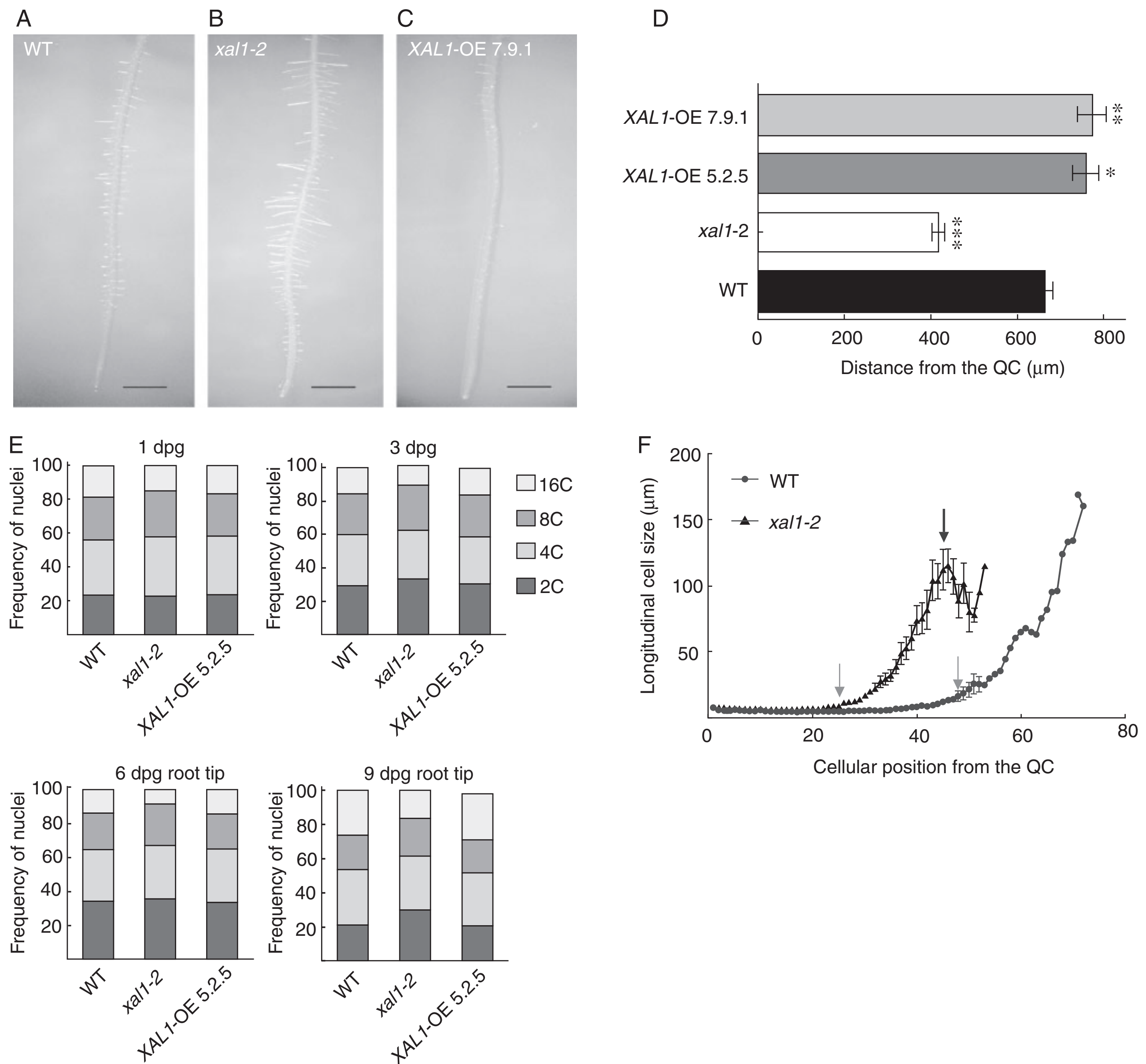

Fig. 4. XAL1 regulates cell transitions to differentiation in Arabidopsis root. (A–C) *xal1-2* (B) has larger root hairs and these appear at shorter distances from the root-tip, while *XAL1*-OE 7.9.1 (C) has shorter root hairs that first appear at more distant positions with respect to the QC in comparison to WT (A). Roots of 5 dpg plants. Scale bars = 1 cm. (D) Distance to the first root hair from the QC in WT, *xal1-2* and both overexpression lines, 5.2.5 and 7.9.1, in 5 dpg seedlings. Data correspond to mean ± s.e. and statistically significant differences (*$P < 0{\cdot}05$, **0·01, ***0·001) were obtained with the Kruskal–Wallis test ($n = 15$). (E) Ploidy distribution of nuclei DNA content of WT, *xal1-2* and *XAL1*-OE 5.2.5 from 1 and 3 dpg whole roots and 6 and 9 dpg root-tips (1 cm long). (F) Cell size profiles of cortex root cells of *xal1-2* and WT roots from the QC to the first root hair in 5 dpg roots. Grey arrows show the point at which cells start to elongate, and the black arrow indicates the point at which cortex cells stop growing. The last point in the curve corresponds to the first cell that presents a root hair. Data correspond to mean ± s.e. ($n = 20$).

such as *rhl1*, *rhl2*, *bin4* and *mid* showed very few root hairs and less developed trichomes than wild-type plants (Sugimoto-Shirasu *et al.*, 2002; Kirik *et al.*, 2007). As endoreplication and higher ploidy levels have been related to cell differentiation as in trichome cells (Walker *et al.*, 2000; Pattanaik *et al.*, 2014), it is surprising that the *xal1* mutant with low ploidy levels is able to develop root hairs that are rather longer, and not shorter, than wild-type roots. Also, it was unexpected that in the OE lines ploidy levels were not significantly different from those observed in wild-type roots, although as XAL1 is a MADS-domain transcriptional factor it is possible that its function requires the participation of additional MADS-domain proteins. This is the case in floral organ development during which the overexpression of single MADS-domain proteins is not sufficient to cause the conversion of leaves into floral organs (Honma and Goto, 2001). Overexpression of *XAL1* is probably not sufficient to alter cell growth and endoreplication.

In conclusion, the data presented in this study suggest that XAL1 participates in the temporal pattern of cell-fate decisions, particularly during the elongation/differentiation transition,

probably by affecting endoreplication maintenance and compromising the final cell size of cortical cells (Tapia-Lopez *et al*., 2008).

Several signals including plant hormones alter root development, particularly proliferation profiles along the root, and the rate and distance from the QC at which cells transit from the proliferation to elongation/differentiation zones are affected by auxin and cytokinin concentrations, at least (Dello Ioio *et al*., 2007, 2008; Moubayidin *et al*., 2010; Takahashi *et al*., 2013). Indeed, auxins and cytokinins have been associated with all or most of these phenotypes in Arabidopsis root development (Dello Ioio *et al*., 2008; Moubayidin *et al*., 2010; Su *et al*., 2011). The *xal1* mutant and *XAL1*-OE lines have similar phenotypes to those described above. We also know that *XAL1* is positively regulated by auxins (Tapia-Lopez *et al*., 2008) and the *PLT1* gene is induced by XAL1 (Fig. 2C). Hence, these hormone networks and the *XAL1* regulatory module are probably interconnected and together underlie proper root development.

XAL1 *participates in cell division regulation at the SCN*

The *XAL1* loss-of-function mutant (Tapia-Lopez *et al*., 2008) and OE lines presented abnormal stem-cell proliferation (Fig. 3). Therefore, it is likely that *XAL1* is a component of the complex regulatory network that underlies stem-cell divisions in the Arabidopsis roots. Among other transcription factors, the *PLT* genes are important components of such a network (Azpeitia *et al*., 2013; Davila-Velderrain *et al*., 2014*a*, *b*). *PLTs* are necessary for stem-cell maintenance and cell division in the root (Aida *et al*., 2004; Galinha *et al*., 2007) and also their proteins accumulation level define the location of developmental zones (proliferation, elongation and differentiation) (Mahonen *et al*., 2014). Interestingly, here we have shown that XAL1 is a positive regulator of *PLT1* (Fig. 2C). PLT1 is an AP2 transcription factor family member that regulates *CYCB1;1* transcription during root development (Aida *et al*., 2004). *PLT1* presents nine CArG boxes in its regulatory region that suggest that XAL1 could directly bind the *PLT1* promoter [plant *cis*-acting regulatory DNA elements (PLACE), http://www.dna.affrc.go.jp/PLACE/]. Interestingly, the *plt1 plt2* double mutant has reduced cell division rate in the root meristem and also shows diminished levels of *CYCB1;1::GUS* expression (Galinha *et al*., 2007), as was found for *xal1-2*. Furthermore, in a recent genomic study that inferred regulatory networks for Arabidopsis root from available microarray data, we found interactions between *XAL1* and *PLT* genes (Chavez Montes *et al*., 2014).

In conclusion, our study further documents that *XAL1* is an important component of the network underlying cell proliferation and elongation/differentiation transitions and overall cell proliferation at the root stem-cell niche. Moreover, *XAL1* participates in the maintenance of cell endoreplication. The results presented here for this MADS-box factor together with previous studies (Tapia-Lopez *et al*., 2008; Chavez Montes *et al*., 2014) suggest that the regulatory networks in which this, and probably other MADS-domain proteins participate, underlie all such dynamics. The role of XAL1 in cell proliferation, cell-cycle duration, cell transition to elongation/differentiation and endocycle performance may be explained, at least in part, by its regulatory role of some relevant cell-cycle components.

SUPPLEMENTARY DATA

Supplementary information is available online at www.aob.oxfordjournal.org and consist of the following. Fig. S1: *pCYCB1;1::GUS* mitosis marker in WT, *xal1-2* and *XAL1*-OE 5.2.5 line at 1, 3 and 7 dpg. Fig. S2: *XAL1* mRNA accumulation in *XAL1*-overexpression lines. Fig. S3: Cell production rate and mature cortical cell length in *XAL1* mutant and overexpression lines. Table S1: Primer sequences used for quantitative and semi-quantitative RT-PCR.

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