An AGAMOUS-Related MADS-Box Gene, XAL1 (AGL12), Regulates Root Meristem Cell Proliferation and Flowering Transition in Arabidopsis¹[W][OA]

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MADS-box genes are key components of the networks that control the transition to flowering and flower development, but their role in vegetative development is poorly understood. This article shows that the sister gene of the AGAMOUS (AG) clade, AGL12, has an important role in root development as well as in flowering transition. We isolated three mutant alleles for AGL12, which is renamed here as XAANTAL1 (XAL1): Two alleles, xal1-1 and xal1-2, are in Columbia ecotype and xal1-3 is in Landsberg erecta ecotype. All alleles have a short-root phenotype with a smaller meristem, lower rate of cell production, and abnormal root apical meristem organization. Interestingly, we also encountered a significantly longer cell cycle in the strongest xal1 alleles with respect to wild-type plants. Expression analyses confirmed the presence of XAL1 transcripts in roots, particularly in the phloem. Moreover, XAL1::β-glucuronidase expression was specifically up-regulated by auxins in this tissue. In addition, mRNA in situ hybridization showed that XAL1 transcripts were also found in leaves and floral meristems of wild-type plants. This expression correlates with the late-flowering phenotypes of the xal1 mutants grown under long days. Transcript expression analysis suggests that XAL1 is an upstream regulator of SOC, FLOWERING LOCUS T, and LFY. We propose that XAL1 may have similar roles in both root and aerial meristems that could explain the xal1 late-flowering phenotype.

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Normal morphogenesis depends on the equilibrium between cell proliferation and differentiation (i.e. cellular homeostasis), whereas transcriptional regulatory networks reliably translate genetic information to yield specific and complex multicellular patterning. In both animals and plants, elegant models of pattern formation have suggested the existence of mechanisms that determine developmental identities in precise manners (Coen and Meyerowitz, 1991; Lawrence and Morata, 1994). Dynamic regulatory network models have substantiated the existence of these mechanisms (von Dassow and Odell, 2002; Espinosa-Soto et al., 2004). Only recently, molecular links between mechanisms that underlie cell-type specification and cell-cycle regulation have been demonstrated (Caro et al., 2007). The MADS-box gene family encodes a large variety of transcriptional regulators of plant and animal development (Messenguy and Dubois, 2003). These transcription factors have been classified into two classes based on sequence relationships and structural features (type I and II lineages) that should have derived from at least one ancestral duplication before the divergence of animals and plants (Alvarez-Buylla et al., 2000b).

Therefore, plant type I is closely related to the animal SRF factors, whereas plant type II is more similar to the MEF type of animals in their MADS domains than to
plant type I. However, type II MADS-domain proteins of plants have three domains (I, K, C) in addition to the MADS DNA-binding domain: a small I domain that links the MADS with the dimerization K domain and the COOH domain (Riechmann and Meyerowitz, 1997).

Plant MIKC genes have been mostly characterized as regulators of the transition to flowering (Samach et al., 2000) and flower, fruit, and seed development (Bowman et al., 1991; Gu et al., 1998; Ferrandiz et al., 2000; Nesi et al., 2002; Pinyopich et al., 2003). They are fairly specific meristem- (Mandel et al., 1992; Bowman et al., 1993), cell- (Liljegren et al., 2000), or organ-identity (Yanofsky et al., 1990; Pelaz et al., 2000) genes. However, genome-wide studies suggest that most MADS-box genes are expressed at different stages of the plant’s life cycle and in a variety of organs and cell types (Kofuji et al., 2003; for review, see Rijpkema et al., 2007), suggesting that these genes may have developmental roles that affect multiple tissues and organs.

Given the high sequence conservation of MADS domains of plant and animal proteins within each lineage (I and II), we hypothesized that some of their functions may also have been conserved. Animal MEF-related MADS proteins have been implicated in regulation of cellular homeostasis and linked to cell-cycle control (Lazaro et al., 2002). Therefore, we proposed that some plant MIKC genes might be important modulators of cell proliferation versus differentiation decisions. Moreover, quantitative cellular analyses of MADS-box mutants may help to further understand the role of these genes in various plant developmental processes.

We have focused on MADS-box genes expressed in the root because this organ is transparent and simple at the cellular level, enabling quantitative analyses of cell dynamics (Dolan et al., 1993; Malamy and Benfey, 1997). Indeed, the root has become a very useful system for unravelling general features of multicellular developmental mechanisms (Benfey and Scheres, 2000; Chapman et al., 2003; Wildwater et al., 2005), and specifically for understanding the links between cellular dynamics and cell-type specification during normal morphogenesis of a complex organ in vivo (Sabatini et al., 2003; Wildwater et al., 2005; Caro et al., 2007). Some components of the molecular mechanisms involved in stem-cell niche patterning and behavior (Sablowski, 2004a; Sarkar et al., 2007), as well as in the patterns of cell proliferation along morphogenetic gradients, which in the root are importantly determined by auxins, have been characterized (Sabatini et al., 1999; Galinha et al., 2007; Grieneisen et al., 2007).

In this study, we report the characterization of AGAMOUS (AG)-related genes that are specific for reproductive tissues. In contrast, XAL1 was characterized as a root-specific gene (Rounsley et al., 1995). Here, we confirm that XAL1 is indeed expressed in roots, but we report its expression also in aerial organs. Our data suggest that XAL1 is an important regulator of cell proliferation in the root. XAL1 mutant alleles have short roots with an altered cell production rate, meristem size, and cell-cycle duration, and thus XAL1 is the first MADS-box gene that is shown to be involved in cell-cycle regulation. Auxins have been implicated in cell-cycle regulation (Himanen et al., 2002; Vanneste et al., 2005) and our data interestingly show that XAL1 is induced by auxins. On the other hand, xal1 alleles are also late flowering and our data suggest that XAL1 could be an important promoter of the flowering transition through up-regulation of SOC, FLOWERING LOCUS T (FT), and LFY.

RESULTS

XAL1, a Sister Gene of the AGAMOUS MADS-Box Clade, Is an Important Regulator of Root Development

Sequence analysis of XAL1 indicated that this gene is a member of the MADS-box transcription factor family (Fig. 1A) and recent phylogenetic analyses suggested that XAL1 is sister to the rest of the AG-related genes (Martínez-Castilla and Alvarez-Buylla, 2003; Parencova et al., 2003). However, contrary to the other members of the AG clade, the expression of XAL1 is not restricted to reproductive organs because it is strongly expressed in roots (Rounsley et al., 1995; Burgeff et al., 2002). To further characterize this gene at the functional level, we isolated three xal1 mutant alleles (Fig. 1B). The xal1-1 allele has an En-1 transposon insertion (Baumann et al., 1998) in the first exon of XAL1 and the xal1-2 allele is a T-DNA insertion in the second intron (see “Materials and Methods”), both in the Col-0 background. The third allele, xal1-3, is in the Ler background and is a stable transposon mutant allele with the insertion at the end of the fourth exon (Fig. 1B).

In all three mutant alleles, the primary root was shorter than in wild-type plants. xal1-1 seedlings showed a root length intermediate between wild type and xal1-2 and xal1-3 (Fig. 1B), probably due to somatic reversion of this unstable transposon allele that occurred after several generations. We performed northern-blot and reverse transcription (RT)-PCR to corroborate XAL1 mRNA levels in roots of the three mutant alleles. RT-PCR detected low expression of XAL1 in the xal1-1 allele, which correlates with its intermediate phenotype, whereas the other two alleles had no expression of XAL1 mRNA (Fig. 1C).

To test whether the shorter roots of the three alleles could be due to altered cellular organization at the root tip, we analyzed 20 roots of each mutant allele under a confocal microscope. About 30% of the plants of all three alleles showed abnormal root apical meristem (RAM) organization, with the quiescent center (QC)
and the columella being most affected (see examples in Fig. 2A). In a median optical section, the columella initial cells and QC cells could not be clearly recognized and the general meristem organization resembled an open-type RAM (Baum et al., 2002; Chapman et al., 2003). As a result of this disorganization, the root-cap protoderm initials giving rise to both protoderm (epidermis) and lateral root cap were abnormal in shape or could not be detected. Typical T divisions in the epidermis could be detected only in the distal portion of the RAM. This abnormal organization led to an altered columella cell differentiation. Whereas in wild-type plants these cells usually increase in length in each subsequent tier along the root axis toward the distal root end (Fig. 2A), in the affected xal1 plants the columella cells in the root cap were of similar size along the root axis, being almost isodiametric rather than elongated as in wild-type plants (Fig. 2A; data not shown).

To further understand the observed shorter root phenotypes, we undertook quantitative cellular analyses of all xal1 alleles. We have set up a protocol to document a series of cellular parameters geared to establish the role of root MADS-box or other types of genes in cellular homeostasis using the root as a study system (see “Materials and Methods”; Supplemental Table S1). These analyses revealed that all three alleles have a shortened meristem with a significantly lower rate of cell production, and xal1-2 and xal1-3 have longer cell-cycle duration than in wild-type plants (Fig. 2B). In all cell parameters quantified, xal1-1 showed milder phenotypes than xal1-2 and xal1-3 alleles (Fig. 2B; Supplemental Table S1). Therefore, XAL1 constitutes the first MADS-box gene that affects cell-cycle duration and for which quantitative cellular data have been put forward to evaluate the role of these genes in regulating cell proliferation within the RAM.

Given that xal1 mutants have significantly affected rates of cell production and cell-cycle duration, as well as an altered apico-basal pattern of cell behavior, XAL1 could be regulated by auxin or XAL1 could mediate responses to auxin in the root. Gradients and movement in the root of this plant hormone are sufficient to guide root growth by affecting cell behavior in a dose-dependent fashion (Sabatini et al., 1999; Galinha et al., 2007; Grieneisen et al., 2007). Maximal auxin levels maintain cell quiescence, intermediate levels promote cell proliferation, and lower levels induce cell elongation and differentiation (Galinha et al., 2007; Grieneisen et al., 2007).

**XAL1 Is Expressed in the Phloem Tissue and XAL1::GUS Is Positively Induced by Auxins**

To test whether XAL1 responds to auxin levels, we constructed transgenic lines with a 2.8-kb XAL1 promoter region driving the expression of GFP (XAL1::GFP; Fig. 3A) and GUS (XAL1::GUS; Fig. 3B). In the root of 8-d-old plants, GUS expression was detected in the vascular cylinder after 24-h staining, starting from
the elongation zone at the level where no signs of protoxylem differentiation were as yet detectable (Fig. 3B; data not shown). XAL1 promoter activity in the differentiation zone was associated predominantly with protophloem cells (Fig. 3B). These results were confirmed with independent XAL1::GFP transgenic lines, which also reported the expression of the XAL1 promoter in the root phloem in an identical pattern observed in XAL1::GUS lines (Fig. 3A). Additionally, 6.8-kb promoter constructs, as well as mRNA in situ hybridization (data not shown), revealed expression in the phloem. However, in situ data (Burgeff et al., 2002) also showed expression of XAL1 in the root meristem that could not be recovered in the lines of these constructs, probably due to the absence of the second regulatory intron.

During lateral root formation, XAL1::GUS expression became visible only after root emergence, and the pattern was similar to that observed in the primary root (Fig. 3, C and D). This pattern of GUS activity driven by the XAL1 promoter correlated well with a significant reduction also in lateral root length of the xal1-1 plants compared to the wild-type plants (Fig. 3E).

Indole-3-acetic acid (IAA) treatment clearly induced GUS activity driven by the XAL1 promoter (Fig. 3F). Interestingly, GUS expression was intensified only in the phloem tissue (Fig. 3G, left). In contrast, the DR5(7X)::GUS line in the wild-type background (Ulmasov et al., 1997) showed an expanded GUS activity domain that was found in all cell types when the roots were treated with auxins (Fig. 3G, right).

**XAL1 Is a Positive Regulator of Flowering Transition That Responds to Photoperiod and Up-Regulates SOC1, FT, and LFY**

While analyzing the xal1 mutants, we realized that the plants were late flowering (Fig. 4A) and we de-
cided to pursue this phenotype and explore whether XAL1 was expressed in aerial tissues. Indeed, in situ hybridization of XAL1 mRNA revealed expression in floral meristems and also in vascular tissues in leaves (Fig. 4B). Detailed analyses of GUS activity in flower sections demonstrated that XAL1::GUS was specifically expressed in young flower meristems, subsequently becoming restricted to the nectaries (Fig. 4C), which contain phloem cells (Baum et al., 2001).

We further characterized the late-flowering phenotype of the xal1-1 and xal1-2 mutants both in the Col-0 background. The most striking characteristic of these mutants was the significant delay in flowering time measured by the bolting time and the total number of rosette leaves observed under long-day (LD) photoperiods (16 h/8 h) in comparison to wild-type plants (Fig. 4D).

Flowering time is regulated in Arabidopsis (Arabidopsis thaliana) by a network of signaling elements that can be assigned to at least four different pathways (Boss et al., 2004): one that promotes flowering in response to LD photoperiods, one that is essential for flowering under noninductive short-day condition (SD) and depends on the plant hormone GA, one that operates both under LD and SD conditions (also called autonomous pathway), and one that regulates flowering time in response to vernalization (Blazquez et al., 1998; Koornneef et al., 1998b; Blazquez and Weigel, 2000; Putterill et al., 2004). In our experiments, both xal1 mutants flowered almost concurrently as wild-type plants under SD conditions (Table I). Moreover, vernalization or GA3 application rescued the flowering-time defects of xal1 plants to the same extent as in wild-type plants under LD photoperiods (Table I). Thus, XAL1 does not seem necessary for the integrity of the autonomous, GA, or vernalization pathways, but seems to be specifically necessary for the correct functioning of the photoperiod flowering pathway (Koornneef et al., 1998a; Imaizumi and Kay, 2006).

To confirm a possible genetic interaction between XAL1 and previously characterized genetic components of the photoperiod and other integrators of flowering transition pathways (Reeves and Coupland, 2000; Moon et al., 2003), we analyzed mRNA expression of a number of genes known to be key regulators of flowering transition (Fig. 4E). First, we confirmed that XAL1 mRNA levels were reduced in the shoot of both mutants. Indeed, xal1-1 has drastically reduced levels of expression and in xal1-2 we were unable to detect any mRNA expression (Fig. 4E). Interestingly, the flowering promoters FT, SOC, and LFY were clearly reduced at the mRNA level with respect to wild type in both xal1-1 and xal1-2 mutant backgrounds (Fig. 4E). In contrast, CONSTANS (CO) and GIGANTEA (GI; data not shown), which are upstream regulators in the photoperiod pathway (Mouradov et al., 2002), did not show significant alterations in mRNA expression in the xal1 mutants in the Col-0 background. Furthermore, XAL1 is down-regulated in the co-1 background, although it is not totally repressed (Supplemental Fig. S1). On the

Figure 3. XAL1 phloem expression is induced by auxins. A, Confocal image of an XAL1::GFP line taken at the protophloem plane, counterstained with propidium iodide. B, Transverse section of root XAL1::GUS line after GUS staining, counterstained with ruthenium red. C and D, XAL1::GUS expression in two different stages of lateral root development. E, Lateral root length along the primary root axis of the wild type (wt) and xal1-1 allele (n = 15 plants; bars = ss). F, XAL1::GUS expression without (-IAA) and with (+IAA 2 μM). G, IAA-induced phloem GUS activity driven by the XAL1 promoter (left) compared to the broad expression of the DR5::GUS line (right), after they were both treated with IAA (2 μM).
Figure 4. Flowering phenotype of xal1-1 and xal1-2 mutants and XAL1 role in the photoperiod pathway. A, Late-flowering transition phenotype of the xal1-1 mutant compared to wild-type plants. Both plants were 32 d old. B, XAL1 mRNA in situ hybridizations. XAL1 expression (arrows) in vascular tissue (v) of a 20 d after planting (DAP) vegetative shoot transverse section (top); in the inflorescence meristem (IM) longitudinal section (middle); and in the gynoecium (g) and anthers of a floral meristem (bottom). C, GUS expression in a floral bud longitudinal section (middle); and in the gynoecium (g) and anthers of a floral meristem (bottom). D, Comparative transcript accumulation of genes that participate in the photoperiod and integrative flowering pathways. Gene expression levels were analyzed in the shoots of 14-d-old seedlings of wild type and xal1-1 and xal1-2 alleles compared to wild-type plants (wt). E, Comparative transcript accumulation of genes that participate in the photoperiod and integrative flowering pathways. Gene expression levels were analyzed in the shoots of 14-d-old seedlings of wild type and xal1-1 mutants by RT-PCR. TUBULIN was included as a constitutive control. A to E, Plants were grown under LD photoperiods.

other hand, FLC, which is a flowering repressor and acts over FT and SOC (Michaels and Amasino, 1999; Searle et al., 2006), showed a slight up-regulation with respect to wild-type plants in both of the xal1 mutants studied. The latter results also correlate with the late-flowering phenotypes of these xal1 alleles.

DISCUSSION

We have shown here that the Arabidopsis MADS-box gene, XAL1, is required for normal root development and proper flowering transition based on mutant phenotypes of two alleles in the Col-0 background and one allele in the Ler background. These alleles were named here xantal1-1, xantal1-2, and xantal1-3 due to their slow-growing root and late-flowering phenotypes. These results were unexpected considering that XAL1 is a sister gene to the AG-related genes that are specific for reproductive tissues, and that most previously characterized MADS-box genes cluster in phylogenetic clades of genes with similar functions and expression patterns during flower, ovule, or carpel development (Rounsley et al., 1995; Alvarez-Buylla et al., 2000a). Nonetheless, previous studies for XAL1 had already suggested that this gene could function in root development due to its high and apparently specific expression in roots (Rounsley et al., 1995; Burgeff et al., 2002). In this study, we have confirmed that XAL1 is indeed expressed in roots, but we show that it is also expressed in aerial tissues prior to the transition to flowering and within floral meristems. In accordance with this pattern of expression, XAL1 is also important for flowering transition.

Functional involvement in more than one tissue or developmental stage might be more common among MADS-box genes than originally believed based on the characterization of the flower-specific MADS-box genes of the A, B, and C functions (Coen and Meyerowitz, 1991). Indeed, recent studies have shown that most genes of this family are expressed in several plant tissues, organs, and developmental stages (Kofuji et al., 2003; Pirenicoava et al., 2003; Schmid et al., 2005). Other studies suggest that MADS-box functional specificity may depend on combinatorial protein-protein interactions (Egea-Cortines et al., 1999; Honma and Goto, 2001; de Folter et al., 2005; Kaufmann et al., 2005; Gregis et al., 2006; Sridhar et al., 2006), rather than on specific spatiotemporal expression patterns for each gene determined at the transcriptional level, as had been suggested before (Savidge et al., 1995; Alvarez-Buylla et al., 2000a).

XAL1 Is an Important Regulator of Cell Proliferation in the Root Meristem

In the root axis, three main zones with contrasting cell proliferation patterns can be distinguished: the RAM, where active cell proliferation takes place from the stem cell niche established around the QC or organizer, and two zones where cells are not proliferating, namely, the elongation and the differentiation zones (Fig. 5; Dolan et al., 1993; Ioio et al., 2007). The data summarized in this article suggest that XAL1 is an important component of the molecular mechanisms controlling cell proliferation in the root. Consequently, the loss-of-function alleles analyzed for this gene show clear spatial alterations of cell behavior along the longitudinal axis of the Arabidopsis root with respect to wild-type plants. Our data suggest that this phenotype is indeed due to the lack of XAL1 because we observed complementation to wild-type root phenotypes using a 35S::XAL1 construct plasmid transformed into xal1-1 and xal1-2 (data not shown).
Drastically diminished levels of XAL1 expression were correlated with altered cellular organization of the RAM, but only in one-third of the analyzed plants for the three xal1 alleles. In these cases, we observed periclinal divisions of the QC early in root development and also lateral expansion of columella cells. However, all xal1-2 and xal1-3 mutant roots were shorter and had a decreased cell production rate, shorter elongated cells, and a significantly longer cell cycle that correlated with smaller meristems. Therefore, the altered cellular patterns at and around the QC in the affected plants are likely to be a consequence rather than a cause of the diminished cell production rates in the root meristem. In any case, these data suggest that type II plant MADS-box genes could be directly involved in cell-cycle regulation. The punctate pattern of mRNA in situ expression revealed for XAL1 in the root meristem tissues is also suggestive of a correlation of this gene expression with cell-cycle stage (Burgeff et al., 2002). In addition, XAL1 is also involved in the regulation of cell elongation. However, this effect is apparently masked in the weaker xal1-1 allele (data not shown; Supplemental Table S1; Figs. 2 and 5).

Future studies should further pursue the role of XAL1 in the molecular networks controlling cell proliferation, elongation, and differentiation. Some components of such networks during root development have been characterized. SHORT-ROOT (SHR) and SCARECROW (SCR) are required for QC identity and normal root growth in addition to their role in radial patterning (Scheres et al., 1995; Di Laurenzio et al., 1996; Helariutta et al., 2000; Wysocka-Diller et al., 2000; Nakajima et al., 2001, Sabatini et al., 2003). However, because the SHR/SCR pathway specifies the entire layer surrounding provascular tissues in the root, it is necessary, but not sufficient, to define the exact position of the stem cell niche. Auxin is also an important signal of QC establishment and it regulates the SCR and PLETHORA (PLT) genes, which are also necessary for QC determination (Sablowski, 2004b).

WOX5 is also expressed in the QC and this gene seems to be necessary and sufficient for stem cell identity (Sarkar et al., 2007), probably with a more direct function in stem cell signaling, rather than in specifying QC identity. WOX5 protein or, most probably a downstream factor, might move to stem cells to maintain their identity (Sarkar et al., 2007). In contrast to these genes that have been shown to be important in QC specification and root growth, XAL1 does not show a peak of expression in the QC or stem cell niche, but loss-of-function mutants in this gene also show cellular aberrations in this zone and clear alterations in cell proliferation and root growth. This suggests that this MADS-box gene could be itself a non-cell autonomous signal from more differentiated tissues (columella and vascular tissues) or control another non-cell autonomous downstream component, which could also be important for QC and stem cell behavior and thus cell production rate in the root meristem. It will be important to use genetic approaches to test whether XAL1 functions are independent or not of SCR, SHR, and WOX5 pathways.

Our data demonstrate that the cell production rate is lower in xal1 mutants than in wild type, but premature cell differentiation could also contribute to the smaller meristems of xal1 mutants. Interestingly, recent experiments have shown that cytokinins affect cell differentiation and define the root meristem by antagonizing from the transition zone a non-cell autonomous signal that could be auxin (Ioio et al., 2007). Moreover, down-regulation of cytokinins in the vascular tissue is sufficient to enlarge the root meristem by retarding the transition of cells to the elongation and differentiation zones. These results and xal1 data presented here thus suggest that XAL1 could be regulated and/or mediate cytokinin functions. This should be tested with genetic approaches.

### Auxin Up-Regulates XAL1 Specifically in the Root Phloem

Auxin promotes cell elongation, cell-cycle duration, and cell differentiation (Evans et al., 1994; Abel and Theologis, 1996; Himanen et al., 2002; Vanneste et al., 2005). In the root, auxin gradients and movement are sufficient to guide root growth (Sabatini et al., 1999; Galinha et al., 2007; Grieneisen et al., 2007) and affect cell behavior in a dose-dependent fashion (Galinha et al., 2007; Grieneisen et al., 2007). In concordance, auxin response or transport mutants display root-patterning defects and exogenous application of auxin induces ectopic QC and stem cells (Sabatini et al., 1999; Friml et al., 2002). Given that the xal1 mutants analyzed here showed root phenotypes affected in these traits,

| Plant Line | LD | LD + VER | SD | SD + GA3 |
|------------|---------|----------|------|---------|
| Col-0      | 33.1 ± 0.4 (n = 78) | 25.2 ± 0.6 (n = 37) | 70.0 ± 0.7 (n = 38) | 44.0 ± 0.5 (n = 21) |
| xal1-1     | 37.5 ± 0.5 (n = 48) P < 0.0001 | 28.0 ± 1.9 (n = 41) | 68.1 ± 1.9 (n = 18) | – |
| xal1-2     | 37.4 ± 0.4 (n = 70) P < 0.0001 | 26.9 ± 0.5 (n = 46) | 71.7 ± 0.7 (n = 27) | 44.5 ± 0.5 (n = 21) |

**Table 1. Bolting time of xal1-1 and xal1-2 mutant plants compared to wild type (Col-0) at different flowering-transition pathways**

Days after sowing are expressed as mean ± s.e and results for LD photoperiod are statistically significant. Flowering-time measurements and conditions for LD and SD photoperiods and both of them after vernalization (+VER) and gibberellin (+GA3) treatments, respectively, are explained in “Materials and Methods.”
Figure 5. Model for the role of XAL1 in root and shoot development. The MADS-box gene, XAL1, might mediate auxin participation in the proliferation of the root meristematic cells and the shoot meristem. In the root, XAL1 may also be implicated in cell elongation because the xal1-2 allele has smaller cells than wild type. Auxin may participate in the shoot meristem transition to flowering, mediating light induction of XAL1, which in turn may be an important promoter of downstream regulators in the photoperiod pathway. CO also induces XAL1 expression probably by the classical photoperiod pathway. Solid arrows indicate direct proved regulation and dashed arrows suggest direct/indirect regulation.

XAL1 could mediate auxin function. Indeed, our data clearly show that XAL1 is up-regulated after IAA treatment within the root phloem tissues, where it is normally and strongly expressed. The XAL1 promoter also responds positively to other auxin analogs, 2,4-dichlorophenoxyacetic acid (2-4D) and naphthaleneacetic acid (NAA; data not shown). Our results thus suggest that there is a phloem-specific factor that responds to auxins and that is required for XAL1 transcriptional up-regulation within these tissues or that XAL1 is itself an auxin-responsive factor. The latter is supported by the presence of several auxin response elements, TGA (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and SAUR boxes (http://www.dna.affrc.go.jp/PLACE; Higo et al., 1999) in the promoter of XAL1.

XAL1 could also be important for phloem cellular patterning. Careful examination of the phloem in the xal1 mutants indicated, however, that XAL1 does not have a key role in the morphogenesis of this tissue on its own because procambial establishment and vascular cell identity in the root are not affected in xal1 mutants in comparison to wild-type plants.

Downstream molecular mechanisms that integrate the cellular effects of auxins and other plant hormones, such as cytokinins, in different spatiotemporal domains during root development are not fully understood. Our data suggest that XAL1 could be one component of such mechanisms. Interestingly, it has recently been suggested that the PLT1 and PLT2 genes, which depend on auxin and auxin response factors for expression, could be the read-out of the root auxin gradient (Galinha et al., 2007). The XAL1 role on cell behavior along the root axis could be related to PLT1 and PLT2 function or it could be part of an independent mechanism. The latter seems to be the case given that XAL1 expression does not overlap with that of the PLT1 and PLT2, which have a gradient-type expression pattern similar to that of auxins with a peak of expression at the QC (Aida et al., 2004). However, other PLETHORA (PLT3 and BBM) genes have strong mRNA expression in the columella stem cell layer and the provascular tissues and could partially overlap with XAL1 expression (Galinha et al., 2007).

XAL1 Is a Promoter of the Floral Transition and Participates in the Photoperiod Pathway

Interestingly, XAL1 is not only important for root development, but is also expressed in aerial tissues and is an important component of the photoperiod pathway of flowering transition, functioning as a flowering promoter in Col-0 Arabidopsis (Reeves and Coupland, 2000; Mouradov et al., 2002). The diminished mRNA levels of the three flowering promoters, SOC, FT, and LFY in the xal1-1 and xal1-2 mutant backgrounds are consistent with this interpretation. FT and SOC act as floral integrators of several pathways, whereas LFY is a flower meristem identity gene that positively responds to FT and SOC1 (Blazquez and Weigel, 2000; Ng and Yanofsky 2000; Moon et al., 2003; Corbesier and Coupland, 2006). None of the other three flowering-transition pathways was affected in these mutant alleles (Table I).

In contrast to several key components of the photoperiod pathway (e.g. CO, GI, CRYPTOCHROME2 [CRY2], and FT; Koornneef et al., 1998a; Simpson and Dean, 2002; Komeda, 2004), the xal1 late-flowering phenotype under LD photoperiods can be recovered to a wild-type phenotype following vernalization (Michaels and Amasino, 2000). In agreement with this, the MADS-box flowering repressor FLC (Michaels and Amasino, 1999; Rouse et al., 2002) is up-regulated in xal1 backgrounds. Therefore, our data suggest that XAL1 could be downstream of CO and GI and upstream of SOC, FT, and LFY. However, complementation of co and gi mutants with XAL1 overexpression constructs, and conversely the overexpression of SOC1 in the xal1 mutant backgrounds, should be pursued in the future to confirm the proposed role of XAL1 in the photoperiod pathway.

There are two possibilities to reconcile the root data for the xal1 mutants with their phenotypes in flowering transition. One possibility is that, given the recently proposed role for auxin response factors in flowering (Ellis et al., 2005; Okushima et al., 2005), XAL1 is a mediator of auxin signaling and participates in the regulation of cell behavior in root and shoot meristems, thus altering their transitions (Fig. 5). The second possibility is that XAL1 has different roles in root and aerial meristems as part of different complexes with other MADS-box proteins, or being a downstream component of different signaling mechanisms.

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MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) wild-type, xal1-1, and xal1-2 plants, co-1, and the DR5(3X)::GUS auxin reporter line (Umasonov et al., 1997) are in the Col-0 genetic background, whereas xal1-3 is in the Ler ecotype. Seedlings were grown on vertical plates with 0.2% Murashige and Skoog salts and 1% Suc. Plants were grown in climate chambers at 22°C. The photoperiods (110 μE m⁻² s⁻¹) were established at 16 h of light followed by 8 h of dark for LD photoperiods and 8 h of light followed by 16 h of dark for SD photoperiods.

Identification of Mutant Alleles

The xal1-1 allele was identified by screening for En-1 insertions among a collection of Arabidopsis plants carrying approximately 50,000 independent insertions of the autonomous maize (Zea mays) transposable element (Baumann et al., 1998). The collection was screened in pools using the En-1 transposon primer En205 (5’-AGAAGCAGGGCGGTGAATAGCCA-3’) and the internal XAL1 primers OEA141 (5’-GCTCGTTTCTTCTTGC-3’) and OEA145 (5’-CAATTCATCTTCACCA-3’). The xal1-2 homozygous line was isolated from the Nottingham Arabidopsis Stock Centre T1 generation stock AB409367 (former GK_306410 from the GABI-Kat collection). Plants 100% resistant to sulfadiazine were further confirmed by PCR using the following primers: GT T-DNA (5’-CCACTTGGAGTTGAATAGCAAC-3’) and specific XAL1 primers NASC12-LP (5’-ACCCAAAAGCTCAATCATCAG-3’) and NASC12-RP (5’-CTTCTTTCGGAAACAAATGC-3’). The xal1-3 allele was identified by screening on a two-component system mutagenized collection based on the maize mobile/transposon Spm as described by Speulman et al. (1999).

Microscopy

Plant material for light microscopy was prepared as previously described by Malamy and Benley (1997). Roots were visualized under an Olympus BX60 microscope. Confocal images were acquired on an inverted Zeiss LSM 510 microscope. Confocal images were acquired on an inverted Zeiss LSM 510

Quantitative Analysis of Cellular Parameters of Root Growth

Length of the meristem was determined for the cortex cells as the distance between the root-body/root-cap junction to the level where cells started to elongate, according to Casamitjana-Martinez et al. (2003). The length of the elongation zone was taken as the distance between the proximal meristem border and the location of the most distal root-hair bulge. The average cycle time for cortical cell production in plants growing between 7 to 8 d was done, using the rate of cell production (Ivanov and Dubrovsky, 1997). The duration of the cell cycle (T) was calculated for each individual root using the following equation: T = (ln2 Nc) / V, where Nc is the number of meristematic cells in one file of the cortex, I is the fully elongated cortex cells in the same root, and V is the root growth rate calculated as μm h⁻¹. Nc in 7- and 8-d-old roots (a period during which the rate of root growth was estimated) was similar in both the wild-type and mutant plants, which enabled us to consider root growth to be at steady state and apply the method described above. The rate of cell production was estimated as V(1/2 I)⁻¹ (Baskin, 2000). Statistical Student’s t-test or the Tukey-Kramer test (depending on the sample size) was analyzed by the JMP program, version 5.1.1.

Reporter Lines

For XAL1::GUS and XAL1::GFP constructs, a 2.8 kb or 6.8 kb promoter and the 5’ untranslated region were obtained from a Lambda genomic DNA library and cloned into pGEM-T vector (Promega) as a Sall-Xhol fragment. This fragment was subcloned into the pBI101 binary vector and the mGFP-E-R to generate the XAL1::GUS and the XAL1::GFP lines, respectively. Arabidopsis Col-0 ecotype plants were transformed using the floral-dip method (Clough and Bent, 1998). The transgenic lines were selected based on their kanamycin resistance and the expression analysis was carried out on T2 homozygous lines.

Hormone Treatments and GUS Reaction

XAL1::GUS and DR5(3X)::GUS seedlings were grown for 7 d in hormone-free medium plates and then transferred to growth medium supplemented with 2 μM of the following hormones: IAA, NAA, and 2,4-D for 24 h. After hormone treatment, DR5(3X)::GUS and XAL1::GUS seedlings were subjected to GUS staining during 40 min at room temperature and 5 h at 37°C, respectively. Stained plants were cleared and visualized under a microscope.

In Situ Hybridization and Histochromosomal Analysis

Inflorescence and bud flowers from wild-type and xal1-1 were subjected to in situ hybridization (Drewes et al., 1991). Dioxogenin-labeled XAL1 probes were synthesized using a 113-bp DNA template amplified with 5’-ATAAAACCTGGAAACTTC-3 and 5’-TAAAGTACACACACACTTG-3 primers, cloned in pGEM-T Easy vector. For flower histochromosomal analysis, samples were processed according to the protocol described in Blazquez et al. (1998). For histological root analysis, GUS-stained samples were dehydrated through ethanol/histoclear series until they were substituted with 100% histoclear (National Diagnostics). Finally, material was embedded in Paraplast (Oxford Labware). Transversal sections of 8-μm-thick GUS-positive root samples were counterstained with 0.1% ruthenium red (Scheres et al., 1994).

Expression Analysis by Northern Blot and RT-PCR

Wild-type and mutant seedlings were grown for 14 d on Murashige and Skoog plates under LD conditions. Total RNA was isolated from root or shoot tissue separately using TRIzol reagent (Invitrogen). Semi-quantitative RT-PCR was performed from two different experiments, each time with duplicates. PCR amplification conditions and sequence primers are described in Supplemental Table S2. RNA-flot hybridization was performed with 10 μg of total RNA per lane with a gene-specific 3’ probe, amplified with the following primers: 5’-GGATGTTATGCTTCAAGAAATTC-3’ and 5’-CCAAATTCCATAAAATCCAA-3’. Flowering-Time Measurements

The bolting time was measured as the days after seed sowing required for the stem to develop 1 cm long under either photoperiod condition. Total number of rosette leaves included fully expanded and not fully expanded leaves. For experiments involving vernalization, seeds were plated on Murashige and Skoog medium and kept under dark for 6 weeks at 4°C and then transferred to soil and grown under LD conditions until flowering. To examine GA effects on flowering time, 100 μM GA₄ solution was sprayed once a week starting 30 d after sowing and continued until bolting. Data expressed as mean ± se were analyzed by the JMP program, version 5.1.1.

Phylogenetic Analysis

We performed a Bayesian reconstruction of the phylogenetic relationships among selected type II Arabidopsis MADS-box genes using the whole cDNAs. Bayesian methods with MrBayes according to Huelsenbeck and Ronquist (2001) were used with a Markov chain Monte Carlo exploration of the tree likelihood surface. Four independent Markov chains (three heated) were used according to the Metropolis coupled scheme. The codon substitution model used was that of Goldman and Yang (1994). Four independent runs of 2,500,000 generations each were performed, and every 100 th tree was saved. After checking for Markov chain convergence, we discarded the first 15,000 trees and used the remaining trees to calculate Bayesian posterior probabilities of the clades. Results from every independent run were similar.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NC_003070.5.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. XAL1 expression in co-1 background.

Supplemental Table S1. Quantitative analysis of root development in xal1-2 and xal1-3 strong alleles and their respective control wild-type plants.
Supplemental Table S2. List of the oligonucleotides used for RT-PCR experiments.

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XAL1 Regulates Root Development and Flower Transition

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