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POL and PLL1 phosphatases are CLAVATA1 signaling intermediates required for *Arabidopsis* shoot and floral stem cells

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The post-embryonic development of above-ground tissues in plants is dependent upon the maintenance and differentiation of stem cells at the shoot meristem. The *Arabidopsis* WUSCHEL (WUS) transcription factor establishes an organizing center within the shoot meristem that is essential for specification of stem-cell identity in overlying cells. The CLAVATA (CLV) signaling pathway, including the CLV1 receptor-kinase, promotes the differentiation of stem cells by limiting the *WUS* expression domain, yet the mechanism of CLV signaling is largely unknown. Previously, we have shown that mutations in two protein phosphatases, POLTERGEIST (POL) and PLL1, partially suppress *clv* mutant phenotypes. Here, we demonstrate that POL and PLL1 are integral components of the CLV1 signaling pathway. POL and PLL1 are essential for stem-cell specification, and can also block stem-cell differentiation when overexpressed. We provide extensive evidence that POL and PLL1 act downstream of CLV signaling to maintain *WUS* expression and that they regulate *WUS* at a transcriptional level. Our findings suggest that POL and PLL1 are central players in regulating the balance between stem-cell maintenance and differentiation, and are the closest known factors to *WUS* regulation in the shoot meristem.

KEY WORDS: Organogenesis, CLAVATA, Meristem, Differentiation, *Arabidopsis*

INTRODUCTION

The adult plant body is generated from the continuous and re-iterative organogenesis at two stem-cell containing structures: the shoot and root meristems. The plant shoot meristem is established at the apical end of the embryo, and is responsible for generating all of the organs and lateral meristems found above ground. A functional shoot meristem is maintained through a tightly controlled balance between the proliferation of a group of stem cells residing in the center, and the differentiation of their peripheral and basal progeny cells for the formation of organ primordia and other differentiated tissues. Flower meristems initiated during inflorescence development function similarly to shoot meristems, except for the transient nature of their stem-cell population.

The homeodomain-containing transcription factor WUSCHEL (WUS) is necessary and sufficient within the meristem to specify stem-cell identity. *wus* mutations lead to the loss of shoot meristem stem cells, and *WUS* overexpression gives rise to ectopic stem cells within the meristem (Brand et al., 2002; Gallois et al., 2002; Laux et al., 1996; Lenhard et al., 2002; Mayer et al., 1998; Schoof et al., 2000). *WUS* is expressed immediately basal to the stem cells in what is termed as an 'organizing center' (Mayer et al., 1998).

The CLAVATA (CLV) signal transduction components CLV1, CLV2 and CLV3 act to restrict the domain of *WUS* expression (Brand et al., 2000; Schoof et al., 2000). *CLV1* encodes a receptor-like kinase containing leucine-rich repeat (LRR) motifs, *CLV2* a LRR receptor-like protein and *CLV3* a small secreted polypeptide (Clark et al., 1997; Fletcher et al., 1999; Jeong et al., 1999; Rojo et al., 2002; Ni and Clark, 2006). Recent findings have shown that transient inactivation of the CLV pathway leads to rapid alterations in the expression of meristem regulators, consistent with earlier studies investigating the effect of *clv* mutations on *WUS* expression (Reddy and Meyerowitz, 2005). Despite our improving understanding of the crucial biological role of this signaling pathway in regulating stem-cell specification and differentiation, no downstream signaling intermediates have been definitively identified.

The best candidate for a CLV signaling intermediate is the protein phosphatase kinase-associated protein kinase (KAPP) (Stone et al., 1994; Stone et al., 1998; Williams et al., 1997). KAPP binds, among many receptor-kinases, to CLV1, and both overexpression and co-suppression studies have suggested that KAPP plays a role in repressing CLV1 function (Stone et al., 1994; Stone et al., 1998; Williams et al., 1997). However, neither a definitive genetic study nor a clear mechanism for KAPP function has been reported.

Another potential source of signaling components are modifier mutants. Previous studies have identified mutations in many genes that enhance or suppress the phenotype of *clv* mutants and are potential candidates for signaling intermediates, including *SHOOTMERISTEMLESS*, *ULTRAPETALA*, *REVOLUTA*, *PHABULOSA*, *PHAVOLUTA*, *CORONA*, *PERIANTHIA*, and *WIGGUM/ENHANCED RESPONSE TO ABA 1* (Clark et al., 1996; Fletcher, 2001; Green et al., 2005; Otsuga et al., 2001; Prigge et al., 2005; Running et al., 1998; Running and Meyerowitz, 1996). However, detailed analyses of all of these genes suggest that each acts independently of the CLV signaling pathway.

Two additional genes that modify the *clv* phenotype when mutated are *POLTERGEIST* (*POL*) and *PLL1*, which encode related protein phosphatases. Mutations in either gene provide partial, additive suppression of the stem-cell accumulation of *clv* mutants, raising the possibility that these two genes act redundantly to promote stem-cell identity (Song and Clark, 2005; Yu et al., 2003;

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Yu et al., 2000). However, the *pol pll1* double mutant is seedling lethal, complicating previous efforts to analyze these genes and their potential role in CLV signaling.

In this study, we report a detailed analysis of *POL*, *PLL1, WUS* and *CLV* genetics. We overcome seedling lethality through grafting, and show interactions between mutations in these genes and their overexpression. All of our findings are consistent with a model in which POL and PLL1 act downstream of the CLV proteins, CLV signaling represses POL/PLL1 and POL/PLL1 are required for *WUS* expression.

MATERIALS AND METHODS

Plant materials and growth condition

The *pol-1*, *pol-6* and *pll1-1* mutants were obtained as described previously (Song and Clark, 2005; Yu et al., 2003; Yu et al., 2000). *pol-6 pll1-1*$^+$ plants were isolated from the F_2 progeny obtained from the cross between *pol-6* and *pll1-1*, and, subsequently, *pol-6 pll1-1* double mutants were identified among the progeny of *pol-6 pll1-1*$^+$ plants based on polymerase chain reaction (PCR) genotyping, as described previously (Song and Clark, 2005). Plants were grown as described previously (Song and Clark, 2005). When plants were grown in sterile conditions, seeds were germinated on the half strength of MS media (Sigma) containing 1% sucrose and 0.02% MES solidified with 0.6% agarose after imbibition at 4°C for 2 days following sterilization.

pol pll1 seedlings 4-6 days after germination grown in sterile condition were micro-grafted as described previously (Turnbull et al., 2002). A cotyledon shoulder region of a *pol pll1* seedling was dissected with a razor blade under a dissecting microscope and transferred on top of a wild-type stock that was prepared by being cut at the hypocotyl region. Whether a scion and a stock were positioned correctly along the axis was examined under a dissecting microscope. Grafted plants were moved into soil approximately 5 days after grafting.

Complementation of *pol pll1* with *PLL1* expression and antisense expression of *PLL1*

For the complementation of the seedling-lethal phenotype of *pol-1 pll1-1* double mutants, the *PLL1* cis-elements including 3.0 kb promoter and 0.5 kb terminator were used for the expression of *PLL1* cDNA. *PLL1* cDNA fragment (Song and Clark, 2005) digested with *Sma*I/*Spe*I was first inserted into a pUC19 vector (digested with *Sma*I/*Xba*I) and then the *PLL1* promoter that was digested with *Eco*RI/*Sma*I was inserted in front of the *PLL1* cDNA. Subsequently, the fused fragment was digested with *Eco*RI/*Sal*I and introduced into a pOCA28 vector (Olszewski et al., 1988) containing a *PLL1* 0.5 kb terminator (*Sal*I/*Xho*I) digested with *Eco*RI/*Sal*I. *pol-1 pll1-1*/+ plants were transformed with this construct as described previously (Clough and Bent, 1998). T_1 plants displaying kanamycin resistance were screened and PCR genotyped with PLL1c1/SynLB3 and PLL1c1/PLL1c2 primers to identify plants containing both the *pll1-1* T-DNA insertion and the transgene for complementation (Song and Clark, 2005). Subsequently, their T_2 progeny were PCR genotyped to screen transgenic plants homozygous for *pll1-1*.

For the anti-sense expression of *PLL1*, a full-length cDNA fragment, PCR amplified with PLL1-NSpe/PLL1-C primers (PLL1-NSpe, 5′- AACTAGT-ATGGGAAGTGGATTCTCCTCCT-3′); PLL1-C, 5′-CGCACTAGTTC-AAAGATACTTTCCTGATGAC-3′), was introduced in reverse orientation into a pCB302-3 binary vector containing cauliflower mosaic virus (CaMV) *35S* cis regulatory elements (Xiang et al., 1999). This transgene was introduced into *pol-6* mutants.

Genetic interaction of *POL/PLL1* with *CLV*, *WUS* and the P_{35S}*:CLV3* transgene

clv3-2 pol-6 pll1-1 triple mutants were screened among the progeny of *clv3-2 pol-6 pll1*/+ that were identified from PCR genotyping among the F_2 progeny obtained from the cross between *clv3-2 pol-6* and *clv3-2 pll1* (Song and Clark, 2005). The seedlings of the triple mutants were grown in sterile conditions and grafted as described above, and their floral organ numbers were counted. P_{ER}*:PLL1* and P_{35S}*:PLL1* transgenes (Song and Clark, 2005) were introduced into the *clv1-1*, *clv1-11*, *clv2-1* and *clv3-2* mutant backgrounds by crosses. The individual F_2 progeny seed pools segregating both *clv* and the transgene were selected, and their phenotypes were examined. Scanning electron microscopy (SEM) analysis was performed as described previously (Diévart et al., 2003) using plants at 10 days after germination.

The P_{35S}*:PLL1* transgene was introduced into the *wus-1* mutant background by crosses. The individual F_2 progeny segregating both *wus-1* and P_{35S}*:PLL1* was examined to see whether P_{35S}*:PLL1* alters the floral phenotype of *wus-1*.

F_1 plants were obtained from crosses between a transgenic plant containing P_{35S}*:PLL1* and a transgenic plant containing P_{35S}*:CLV3* (Brand et al., 2000). Plants possessing both P_{35S}*:PLL1* and P_{35S}*:CLV3* were screened among the F_1 plants and their phenotypes were examined to see whether they displayed the Wus$^-$ phenotype. To determine the presence of the P_{35S}*:PLL1* and P_{35S}*:CLV3* transgenes, PCR genotyping was performed using PLL1c1/PLL1c2 primers (PLL1c1, 5′-GTGTTTACTCGAAGAA-GAGACGGA-3′; PLL1c2, 5′-GTGCTCGTTTTTTATTCTTGTTACTTC-3′) and 35Sp1/CLV3r primers (35Sp1, 5′-GATGACGCACAATCCCAC-TA-3′; CLV3r, 5′-TCAAGGGAGCTGAAAGTTGTT-3′), respectively.

Expression of these genes was assessed using primers for *PLL1* (PLL1c1/PLL1c2), *CLV3* (CLV3f, 5′-ATGGATTCTAAAAGCTTTGT-GCT-3′; CLV3r, 5′-TCAAGGGAGCTGAAAGTTGTT-3′) and *TUB* (TUBf, 5′-AGAGGTTGACGAGCAGATGA-3′; TUBr, 5′-CCTCTTC-TTCCTCCTCGTAC-3′).

Expression pattern analyses of reporter genes

P_{WUS}*:GUS* and P_{CLV3}*:GUS* reporter genes (Gross-Hardt et al., 2002; Lenhard et al., 2002) were introduced into mutant backgrounds by crosses. GUS activity was examined in the plants, which were grown in sterile conditions, at 10 days after germination, or in the inflorescence of grafted plants as described previously (Sessions et al., 1999). Tissues were incubated in the staining solution overnight.

Complementation of *pol pll1* by the ectopic expression of *WUS*

For ectopic *WUS* expression, the *pOpL* two-component expression system was used (Moore et al., 1998). *APETALA1* (*AP1*) (Hempel et al., 1998) and *ERECTA* (*ER*) promoters (Diévart et al., 2003) were used to drive the expression of *LhG4* (kindly provided by Michael Prigge, University of Indiana, Indiana, USA). Several independent transgenic lines were screened and crossed with transgenic plants expressing *pOp:WUS* (Schoof et al., 2000) (kindly provided by Thomas Laux) to examine the strength of the promoter based on the phenotypes of F_1 plants. F_1 progeny obtained from the crosses between the selected *AP1:LhG4* line (or *ER:LhG4* line) and *pol-6 pll1-1*/+ were crossed to F_1 progeny obtained from the crosses between *pOp6:WUS* and *pol-6 pll1-1*/+. Among the resulting new progeny, *pol pll1* plants were isolated, grafted and PCR genotyped using gene-specific primers (LhG4f/LhG4r for *AP1:LhG4* and *ER:LhG4*; WUS-N/WUS-C for *pOp6:WUS*) to test for the presence of both transgenes (LhG4f, 5′-TAACGTTATACGATGTCGCAGAG-3′; LhG4r, 5′-CCAAT-GCGACCAGATGCT-3′; WUS-N, 5′-CCCGGGGATGGAGCCGCCA-CAGCATCAG-3′; WUS-C, 5′-GGATCCCTAGTTCAGACGTAGCT-CAAG-3′). *AP1:LhG4* and *pOp6:WUS* transgenes were introduced into the *wus-1* mutant background in a similar manner.

RESULTS

Grafted *pol pll1* tissue phenocopies *wus* mutants

A major obstacle in analyzing POL/PLL1 function in stem-cell specification was the embryo/seedling lethality of the *pol pll1* double mutants (Song and Clark, 2005). We determined that *pol pll1* lethality was largely the result of major defects in basal embryo patterning, and that we could grow *pol pll1* double-mutant tissue by grafting the apical portion of a *pol-6 pll1-1* seedling onto the hypocotyl/root of a wild-type seedling (Fig. 1A-E). Both *pol-6* and *pll1-1* are T-DNA insertion alleles that are putative nulls (Song and Clark, 2005).

The *pol pll1* tissues (Fig. 1F,G) growing in such grafted plants closely phenocopied *wus* mutants (Fig. 1I), with re-iterative termination of shoot apices during vegetative development.

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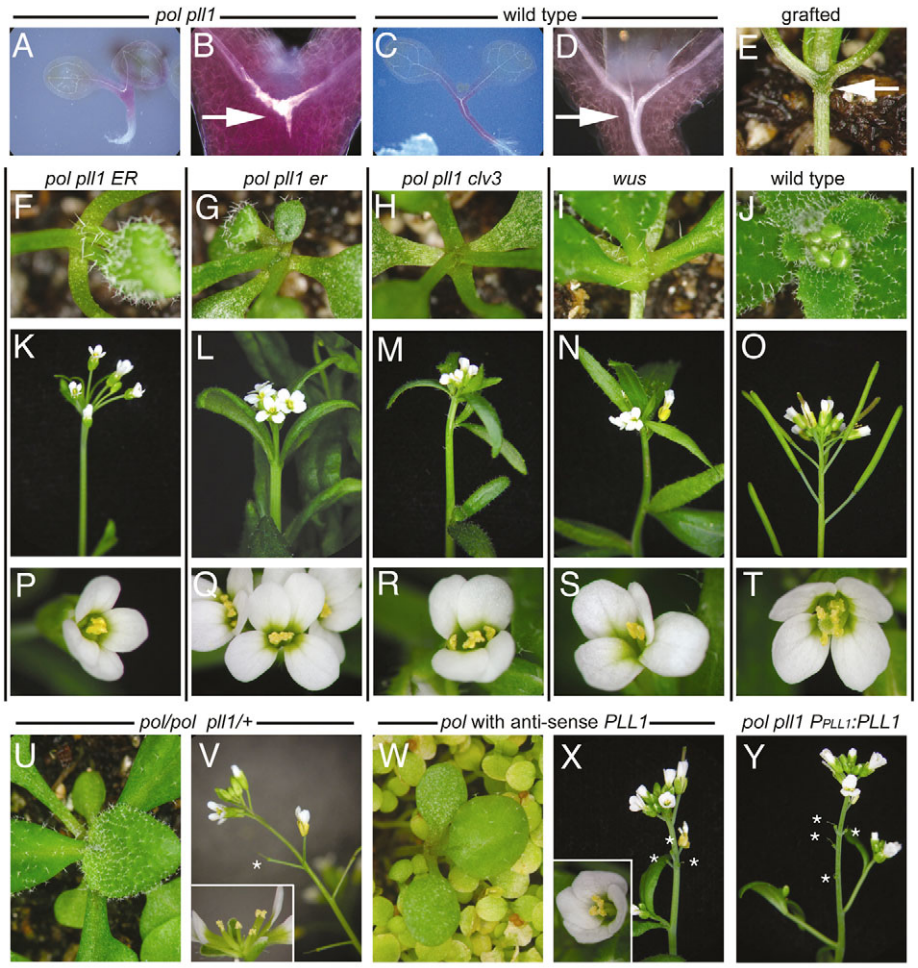

Fig. 1. *pol pll1* mutant tissue phenocopies *wus* mutants. (**A**,**B**) *pol pll1* seedlings were not viable because of major defects in the basal embryo and vascular development compared with wild-type siblings (**C**,**D**). (B,D) Higher magnification of A and C, respectively. (**E**) *pol pll1* apical tissue can be rescued by grafting to the hypocotyl and root of a wild-type seedling. (**F-T**) Grafted *pol pll1* in the *ER*$^+$ (F,K,P), *er*$^-$ (G,L,Q) and *clv3-2* (H,M,R) backgrounds exhibited meristem termination similar to *wus-1* mutants (I,N,S) during vegetative (F-J), inflorescence (K-O) and flower (P-T) development. (J,O,T) Wild-type L*er* shown as a control. Similar meristem termination, albeit with reduced penetrance and expressivity, was observed in *pol/pol pll1/+* plants (**U**,**V**), *pol* mutants expressing antisense *PLL1* (**W**,**X**), and *pol pll1* mutants incompletely rescued by P_{PLL1}*:PLL1* expression (**Y**). Arrows indicate junction of cotyledon vascular elements (B,D) or graft junction (E). Asterisks indicate flowers that developed with reduced or absent gynoecia. Insets in V and X show flowers with reduced or absent gynoecia, respectively, at higher magnifications.

Eventually transitioning to flowering as *wus* mutants do, the *pol pll1* grafted tissue (Fig. 1K,L) gave rise to inflorescence phenotypes similar to *wus* mutants (Fig. 1N), including flowers with reduced numbers of floral organs, presumably as a result of the loss of flower-meristem stem cells (compare Fig. 1P,Q with Fig. 1S). *pol pll1* flowers lacked central carpels and developed reduced numbers of stamens, although the phenotype was slightly less severe than *wus* mutants (Fig. 2). The meristem-termination phenotypes were also observed, albeit less frequently, in *pol/pol pll1/+* plants, in *pol/pol* plants with antisense expression of *PLL1* and in *pol/pol pll1/pll1* plants with incomplete complementation by *PLL1* (Fig. 1U-Y). This indicates that these phenotypes are not related to the grafting technique used to generate *pol pll1* tissue, but are a consequence of reduced POL/PLL1 activity.

pol pll1 is epistatic to *clv3*

The severe loss of meristem activity in *pol pll1* double-mutant tissue allowed us to address whether POL/PLL1 act upstream or downstream of the CLV signaling pathway by generating *clv pol-6 pll1-1* triple mutants. A similar genetic approach was previously used to establish that *WUS* acts downstream of CLV signaling, a finding bourn out by subsequent detailed studies (Laux et al., 1996; Mayer et al., 1998; Schoof et al., 2000). The seedling lethality of *pol pll1* mutants was unaffected by the introduction of the putative null *clv3-2* mutation. The ratio between the viable plants and seedling-lethal plants in the progeny of *clv3-2 pol pll1/+* plants (79:31) and the progeny of *clv3-2 pol/+ pll1* plants (54:19) did not vary significantly from 3:1 based on χ^2 analysis. *clv2-1 pol pll1*, *clv1-7 pol pll1* and *clv1-1 pol pll1* triple mutants also exhibited seedling-lethal phenotypes.

To examine the post-embryonic phenotypes, *clv3-2 pol pll1* mutants were grafted onto the wild-type hypocotyls. *clv3-2 pol pll1* tissue developed in an identical fashion to *pol pll1* double-mutant tissue during vegetative and inflorescence development (Fig. 1H,M). The mean number of organs developing in *clv3-2 pol pll1* flowers was statistically indistinguishable from that of *pol pll1* flowers (Fig. 1R, Fig. 2). These results indicate that *pol pll1* is fully epistatic to *clv3-2*, indicating that POL/PLL1 act downstream of the CLV signaling pathway.

Alteration of stem-cell marker gene expression in *pol pll1* mutants

To determine the nature of meristem defects in *pol pll1* mutants, we crossed *pol pll1* to well-characterized transgenes in which the *CLV3* and *WUS* cis regulatory elements drive β-glucuronidase (GUS) expression (P_{CLV3}*:GUS* and P_{WUS}*:GUS*) and monitored the expression of these key meristem regulatory factors (Gross-Hardt et al., 2002; Lenhard et al., 2002). For P_{WUS}*:GUS*, we observed a clear spot of GUS activity at the shoot meristem in wild-type seedlings, but no signal at all was observed in *pol pll1* seedlings (data not shown). Around the transition to flowering, when *WUS* becomes expressed in the many wild-type flower meristems, we observed occasional punctate spots of *WUS* expression in *pol pll1* plants (Fig. 3A-F). These spots corresponded to expression in internal cell layers

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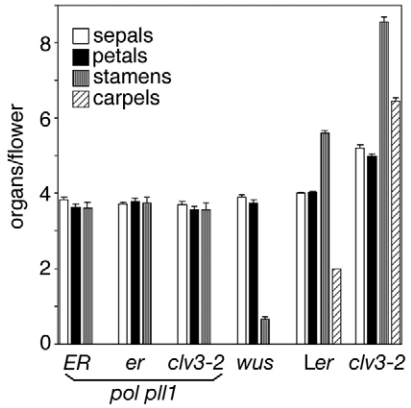

Fig. 2. *pol pll1* mutations are epistatic to *clv3*. Mean number of organs from flowers of grafted *pol pll1* in the *er*⁻, *ER*⁺ and *clv3-2* backgrounds, *wus-1* and *clv3-2* mutants, and wild-type L*er* plants. Error bars indicate standard error of the mean (s.e.m.). At least 50 flowers were counted for each mean.

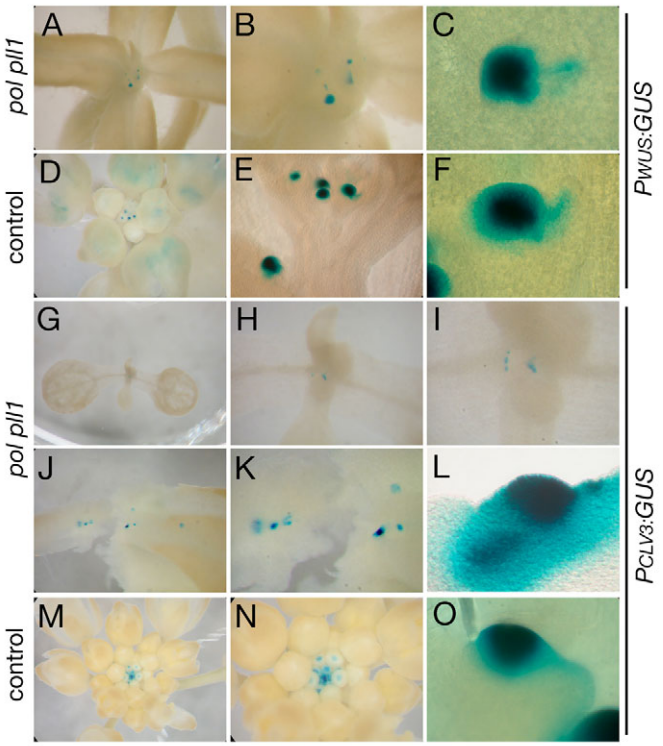

Fig. 3. *pol pll1* tissue initiates, but does not maintain, *WUS* and *CLV3* expression. (A-O) P_{WUS}*:GUS* (A-F) and P_{CLV3}*:GUS* (G-O) signal in *pol pll1* seedlings germinated on MS media (G-I), inflorescence tissue from grafted *pol pll1* plants (A-C,J-L), and phenotypically wild-type siblings (D-F,M-O). Each sample is shown at increasing magnifications to provide context for the location of the signal within the plant.

of what morphologically appeared to be nascent meristems (Fig. 3C,F). When compared to P_{WUS}*:GUS* expression in wild-type plants, the spots in *pol pll1* mutants appeared to correspond to transient apices forming in leaf axils. These results suggest that POL/PLL1 are required for the maintenance, but not the initiation, of *WUS* expression. Because data indicate that CLV signaling is also important for maintenance, but not initiation, of *WUS* expression, these results are consistent with the hypothesis that POL/PLL1 functions within the CLV pathway. If CLV signaling achieves repression of *WUS* through the inhibition of POL/PLL1 activity, one would expect constitutive inhibition of *WUS* in *pol pll1* mutants after initiation.

P_{CLV3}*:GUS* activity was detected in the initiating shoot apical meristem of *pol pll1* embryos; however, the activity was weaker, compared with wild type, and was restricted to the epidermal layer (see Fig. S2 in the supplementary material). Post-embryonically, P_{CLV3}*:GUS* behaved similarly to P_{WUS}*:GUS* in wild-type plants and in *pol pll1* mutants, with punctate spots of P_{CLV3}*:GUS* activity in *pol pll1* mutants in apparent transient shoots (Fig. 3G-O). Similar to wild-type meristems, the P_{CLV3}*:GUS* signal was largely within apical cells layers within these apparently transient meristems (Fig. 3L,O). Both reporter-gene expression patterns and morphology suggest that meristems are initiated but immediately lost in *pol pll1* plants, consistent with hyper-repression of *WUS* after meristem initiation.

Ectopic *WUS* expression bypasses the requirement of POL/PLL1 for stem cells

To definitively test whether the loss of meristem activity in *pol pll1* mutants was the consequence of the loss of *WUS* expression maintenance, we designed a transgene arrangement to determine if ectopic *WUS* expression could bypass the requirement for POL/PLL1. We set expression of *WUS* under the control of the flower-specific *APETALA1* (*AP1*) cis regulatory elements in a transactivation system, in which *AP1*-driven *WUS* expression would only occur in the progeny of plants carrying both the P_{AP1}*:LHG4* driver and the P_{OP6}*:WUS* responder (Hempel et al., 1998; Moore et al., 1998; Schoof et al., 2000). In wild-type plants with P_{AP1}*:LHG4*/P_{OP6}*:WUS* (hereafter referred to as P_{AP1}*:WUS*), flowers underwent extensive meristematic proliferation and eventual carpeloid organ formation (Fig. 4A). *wus* plants with P_{AP1}*:WUS* exhibited defective vegetative development typical of *wus* mutants; however, upon flowering, these plants developed vigorous meristem activity in each flower, giving rise to meristem proliferation and carpeloid organ formation (Fig. 4D). An identical restoration of floral-meristem activity was observed when P_{AP1}*:WUS* was introduced into *pol pll1* grafted tissue, including extensive meristem proliferation and organogenesis (Fig. 4B,C). P_{AP1}*:WUS* in *pol pll1* tissue drove activation of P_{CLV3}, indicating the meristem-like nature of the proliferations (Fig. 4E,F). P_{AP1}*:WUS* did not drive activation of P_{WUS}, suggesting that *WUS* is not under autoregulatory control (Fig. 4G,H). A similar restoration of meristem activity in *pol pll1* mutants was observed when *WUS* expression was driven by the cis regulatory elements for the receptor-kinase *ERECTA* (*ER*) (Diévart et al., 2003; Yokoyama et al., 1998). Using the same transactivation transgene arrangement followed by grafting to generate P_{ER}*:WUS pol pll1* tissue, we observed restoration of meristem proliferation that was less extensive than P_{AP1}*:WUS* and gave rise to more normal floral organs (Fig. 4I,J). Thus, the loss of *pol pll1* meristem activity is directly attributable to the loss of *WUS* expression maintenance, indicating that POL/PLL1 act through WUS to promote stem-cell identity.

Ectopic *PLL1* expression blocks differentiation in *clv* mutants

If POL/PLL1 are indeed targeted for negative regulation by CLV signaling, one would predict that overexpressing POL/PLL1 would enhance *clv* mutants, providing de-repression of excess

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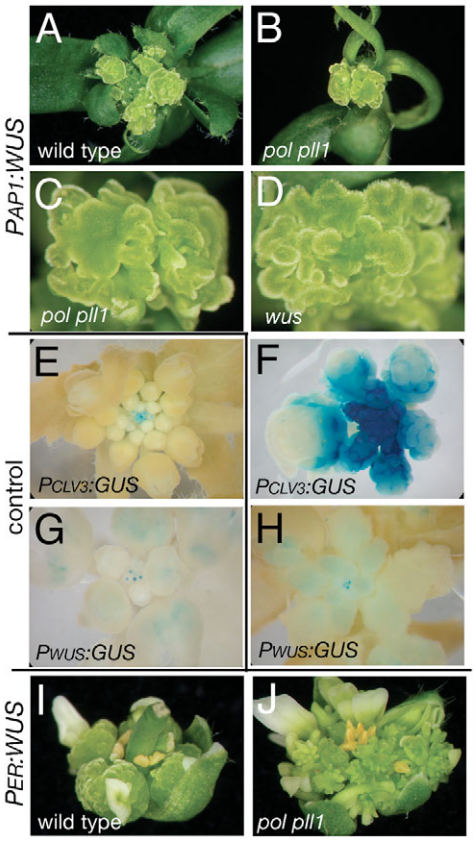

Fig. 4. *WUS* expression rescues *pol pll1* mutants. (**A-D**) Wild-type Ler (A), grafted *pol pll1* (B,C) and *wus-1* mutants (D) expressing P_{AP1}*:WUS* (see text) all developed extensive meristem-like proliferations upon the transition to flowering (C and D shown at higher magnification). P_{CLV3}*:GUS* activity in control (**E**) and P_{AP1}*:WUS* (**F**) plants indicates a stem cell-like character for these proliferations, whereas P_{WUS}*:GUS* activity is similar in control (**G**) and P_{AP1}*:WUS* (**H**) plants. P_{ER}*:WUS* results in similar, but weaker, phenotypes in Ler (**I**) and *pol pll1* grafted (**J**) plants.

POL/PLL1. Given the extensive gene families for *CLV1*, *CLV2* and *CLV3* (Botella et al., 1997; Sharma et al., 2003; Shiu and Bleecker, 2001), and evidence that *clv1* and *clv2* null alleles exhibit rather weak phenotypes (Diévart et al., 2003; Kayes and Clark, 1998), it is unclear whether any *clv* single mutant represents a complete loss of signaling. We have previously shown that *PLL1* overexpression in an otherwise wild-type background gives rise to weak Clv$^-$ phenotypes (Song and Clark, 2005). Here, we drove *PLL1* expression under the control of both *ER* (P_{ER}) and the cauliflower mosaic virus (CaMV) 35S (P_{35S}) cis regulatory elements in the *clv2-1* and *clv3-2* mutant backgrounds, and observed dramatic enhancement of the Clv$^-$ phenotype (Fig. 5). In many cases, the transgenic plants simply produced meristem tissue at the shoot apex, with a complete absence of organ formation over a long developmental window. Some plants senesced and died without producing organs, whereas others developed 'escape' tissue, presumably as a result of transgene repression, that went on to produce a small number of organs after a sustained period without organogenesis (see Fig. S1 in the supplementary material). To determine whether the overproliferating tissue was stem cell-like in nature, we introduced P_{CLV3}*:GUS* and P_{WUS}*:GUS* (Gross-Hardt et al., 2002; Lenhard et al., 2002) into the P_{35S}*:PLL1 clv3-2* background (Fig. 5O,P). In both cases, we observed GUS activity throughout the apex, with the strongest P_{WUS}*:GUS* signal in internal cell layers and the strongest P_{CLV3}*:GUS* signal in the overlying cells. The presence and pattern of both meristem markers, as well as the morphology of these structures, indicate that these accumulated cells are equivalent to the central zone of a wild-type meristem, including stem cells in the top three cell layers. This data also indicate that *clv* mutants have a partial loss of differentiation that is further antagonized by the overexpression of *PLL1*. This is consistent with both a detailed analysis of *clv* mutants, as well as a recent study showing that inducible downregulation of *CLV3* leads to the rapid expansion of the *CLV3* expression domain (Reddy and Meyerowitz, 2005). Indeed, it would be of interest to repeat this sort of analysis in a *PLL1* overexpression background, as P_{35S}*:PLL1 clv3* results in a more complete loss of differentiation than a *clv3* single mutant.

Ectopic stem cells induced by *PLL1* overexpression require *WUS* and suppress *CLV3* overexpression effects

To determine whether the effect of *PLL1* overexpression was entirely dependent upon *WUS*, we assessed the effect of the P_{35S}*:PLL1* transgene in the *wus-1* mutant background. P_{35S}*:PLL1 wus* plants were readily distinguishable from *wus* single mutants during vegetative development because *PLL* overexpression results in altered leaf morphology, namely smaller, rounder leaves (Song and Clark, 2005). Beyond this difference in leaf development, P_{35S}*:PLL1* had no effect that we could detect on the terminated vegetative or inflorescence meristems observed in *wus* mutants (see Fig. S1 in the supplementary material). Similarly, within the flowers, P_{35S}*:PLL1* had no statistically measurable effect on the number of organs formed in *wus* flowers. Most importantly, no increase in the number of stamens or carpels per flower as a result of *PLL1* overexpression was observed (Fig. 6). *wus* is thus fully epistatic to P_{35S}*:PLL1* within the meristem, consistent with the hypothesis that PLL1 acts within the meristem by regulating *WUS*.

Overexpression of *CLV3* leads to shoot- and flower-meristem termination, resulting in plants phenotypically similar to *wus* mutants, albeit less severe (Brand et al., 2000). If CLV signaling acts to repress POL/PLL1 activity, then overexpression of *PLL1* in the P_{35S}*:CLV3* background would be predicted to suppress, at least partially, the meristem termination phenotype. Progeny from a cross between plants carrying the P_{35S}*:CLV3* transgene and plants carrying the P_{35S}*:PLL1* transgene were assayed for the shoot-meristem termination phenotype and genotyped for the presence of the two transgenes. Whereas every plant carrying P_{35S}*:CLV3* alone developed terminated shoot meristems, 80% of the plants carrying both transgenes developed normal shoot meristems (Table 1). Transgene expression analysis indicated that this was not the result of suppression of the P_{35S}*:CLV3* transgene (see Fig. S3 in the supplementary material).

DISCUSSION

Taken together, our analyses strongly suggest that POL/PLL1 are intermediates downstream of the CLV factors in the regulation *WUS* expression and stem-cell differentiation. *POL/PLL1* are required for stem-cell maintenance through their regulation of *WUS* expression. *POL/PLL1* overexpression blocks differentiation and drives stem-cell accumulation, especially in a *clv* mutant background. This would suggest that CLV signaling inhibits POL/PLL1 post-

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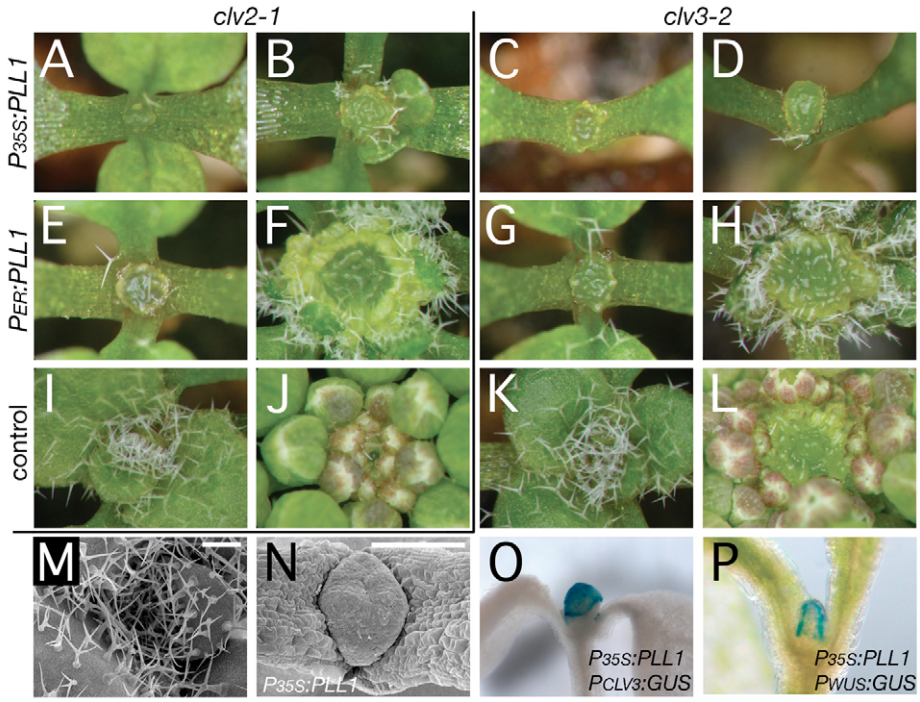

Fig. 5. *PLL1* overexpression in *clv* mutants blocks differentiation. (**A-P**) *clv2-1* (A,B,E,F,I,J) and *clv3-2* (C,D,G,H,K-P) plants carrying P_{35S}*:PLL1* (A-D,N-P), P_{ER}*:PLL1* (E-H) or no transgene (I-M) revealed massive accumulation of stem cells and a block of differentiation resulting from *PLL1* overexpression. (A,C,E,G,I,K,M-O) At 10 days after germination; (B,D,F,H,J,L) 15 days after germination; (P) 6 days after germination. P_{CLV3}*:GUS* (O) and P_{WUS}*:GUS* (P) were expressed throughout the apices of *clv3-2* P_{35S}*:PLL1* plants, indicating stem-cell character. Scale bars: 250 μm.

transcriptionally and that a combination of excess *PLL1* transcription and loss of CLV-mediated inhibition is sufficient to block stem-cell differentiation.

POL/PLL1 regulation of *WUS* appears to be on the level of transcription, as evidenced by the loss of *WUS* transcription in *pol pll1* mutants, as well as by the ability to bypass POL/PLL1 by expressing *WUS* under a different set of cis regulatory elements. The initiation of *WUS* expression in transient shoots in *pol pll1* double mutants, as well as the slightly weaker phenotype of *pol pll1* double-mutant tissue in comparison with *wus* null alleles, reflects that POL/PLL1 regulation of *WUS* expression acts only after meristem initiation. Thus, POL/PLL1 are primarily regulators of the maintenance, not the initiation, of *WUS* expression. This is fully consistent with data on the function of the CLV pathway in regulating *WUS* expression.

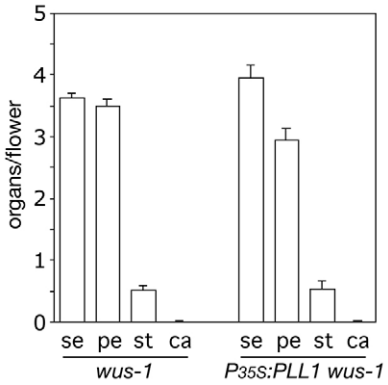

Fig. 6. *PLL1*-overexpression effects are dependent on *WUS*. Mean number of organs from flowers of *wus-1* and P_{35S}*:PLL1 wus-1* plants. Error bars indicate standard error of the mean (s.e.m.). In total, 93 *wus-1* and 39 P_{35S}*:PLL1 wus-1* flowers were assayed. se, sepals; pe, petals; st; stamens; ca, carpels.

It is formally possible that POL/PLL1 act independently on *WUS* rather than acting as intermediates of CLV signaling, but this alternative hypothesis is not supported by the data. First, *pol pll1* mutants do express *WUS*, albeit only in transient shoots. If POL/PLL1 and CLV were acting separately, one would expect the removal of CLV signaling to expand *WUS* expression, and hence alter phenotypes, in *pol pll1* mutants. Therefore, this alternative hypothesis would predict that *clv* should suppress, to some extent, *pol pll1* double-mutant phenotypes. This is especially true in the flower meristem, where we see transient *WUS* and *CLV3* expression in *pol pll1*, and these mutants exhibit flower-meristem defects that are slightly weaker than *wus*. Even here, there is no effect of removing CLV signaling, as evidence by the full epistasis of *pol pll1* to *clv* mutants. In addition, there is an incredible level of dosage sensitivity between *CLV*, *POL/PLL1* and *WUS* in both loss- and gain-of-function situations, suggesting a common pathway.

CLV repression of POL/PLL1 is likely to be post-transcriptional, based on both the broad expression of POL and PLL1 throughout the plant and within the meristem (Yu et al., 2003; Song and Clark, 2005), and on the interaction of *clv* mutants with *PLL1* overexpression. This would be quite typical for receptor signaling intermediates.

Little is known about downstream targets of other receptor kinases. POL/PLL1 share similarity in general pathway function with the BIN2 kinase in BRI1 receptor kinase-mediated brassinosteroid signaling (Li and Nam, 2002). POL/PLL1 and BIN2

Table 1. *PLL1* overexpression suppresses *CLV3* overexpression meristem termination

Genotype of F1 plants (P_{35S}*:PLL1*×P_{35S}*:CLV3*)	n[a]	Wus⁻ phenotypes[b]	Wild-type phenotypes[c]
P_{35S}:CLV3	26	26	0
P_{35S}:CLV3 P_{35S}:PLL1	16	3	13

[a]Number of plants; [b]plants exhibiting shoot termination typical of *wus* mutants; [c]plants with superficially wild-type shoot development.

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appear to be negatively regulated by the corresponding receptor kinase, and said repression allows for signaling to occur. In the case of BIN2 repression, brassinosteroid signaling occurs as the result of the loss of BIN2-mediated phosphorylation and subsequent degradation of a set of transcription factors (Wang et al., 2002; Yin et al., 2002; Zhao et al., 2002). As with BIN2, the exact link between the POL/PLL1 and the upstream receptor kinase remains unclear. Whether CLV1 directly modulates POL/PLL1 activity and what are the targets of POL/PLL1 phosphatase activity are crucial issues to pursue.

pol pll1 mutants have pleiotropic phenotypes, with evidence for POL/PLL1 regulation of basal embryo development, of pedicel development and of leaf vascular patterning (Song and Clark, 2005). These activities may represent the function of POL/PLL1 downstream of other receptor kinases. The redundant nature of many receptor kinases may explain why the corresponding receptors have not yet been identified (Cano-Delgado et al., 2004; DeYoung et al., 2006; Shpak et al., 2004; Zhou et al., 2004).

It is of interest to consider that the CLV1-related BAM receptors in *Arabidopsis* may also utilize POL and related proteins as signaling intermediates. *bam* mutant combinations exhibit many developmental defects, including effects on leaf vascular patterning similar to those that result from the overexpression of *PLL1* (DeYoung et al., 2006; Song and Clark, 2005), raising the possibility that BAM receptors may regulate leaf development by signaling through POL/PLL1 or related phosphatases. The observation that CLV1 can replace BAM function during leaf, stem and floral-organ development suggest a common signaling pathway in each of these different tissues (DeYoung et al., 2006). Given the large receptor-kinase gene family in *Arabidopsis*, it will be crucial to test the association of specific receptors with potential downstream intermediates.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/23/4691/DC1

References

Botella, M. A., Coleman, M. J., Hughes, D. E., Nishimura, M. T., Jones, J. D. and Somerville, S. C. (1997). Map positions of 47 Arabidopsis sequences with sequence similarity to disease resistance genes. *Plant J.* **12**, 1197-1211.

Brand, U., Fletcher, J. C., Hobe, M., Meyerowitz, E. M. and Simon, R. (2000). Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by *CLV3* activity. *Science* **289**, 617-619.

Brand, U., Grunewald, M., Hobe, M. and Simon, R. (2002). Regulation of CLV3 expression by two homeobox genes in Arabidopsis. *Plant Physiol.* **129**, 565-575.

Cano-Delgado, A., Yin, Y., Yu, C., Vafeados, D., Mora-Garcia, S., Cheng, J. C., Nam, K. H., Li, J. and Chory, J. (2004). BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in *Arabidopsis*. *Development* **131**, 5341-5351.

Clark, S. E., Jacobsen, S. E., Levin, J. Z. and Meyerowitz, E. M. (1996). The *CLAVATA* and *SHOOT MERISTEMLESS* loci competitively regulate meristem activity in Arabidopsis. *Development* **122**, 1567-1575.

Clark, S. E., Williams, R. W. and Meyerowitz, E. M. (1997). The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* **89**, 575-585.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735-743.

DeYoung, B. J., Bickle, K. L., Schrage, K. J., Muskett, P., Patel, K. and Clark, S. E. (2006). The CLAVATA1-related BAM1, BAM2 and BAM3 receptor kinase-like proteins are required for meristem function in Arabidopsis. *Plant J.* **45**, 1-16.

Diévart, A., Dalal, M., Tax, F. E., Lacey, A. D., Huttly, A., Li, J. and Clark, S. E. (2003). *CLAVATA1* dominant-negative alleles reveal functional overlap between multiple receptor kinases that regulate meristem and organ development. *Plant Cell* **15**, 1198-1211.

Fletcher, J. C. (2001). The ULTRAPETALA gene controls shoot and floral meristem size in Arabidopsis. *Development* **128**, 1323-1333.

Fletcher, J. C., Brand, U., Running, M. P., Simon, R. and Meyerowitz, E. M. (1999). Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. *Science* **283**, 1911-1914.

Gallois, J. L., Woodward, C., Reddy, G. V. and Sablowski, R. (2002). Combined SHOOT MERISTEMLESS and WUSCHEL trigger ectopic organogenesis in *Arabidopsis*. *Development* **129**, 3207-3217.

Green, K. A., Prigge, M. J., Katzman, R. B. and Clark, S. E. (2005). *CORONA*, a member of the class III homeodomain-leucine zipper gene family in Arabidopsis, regulates stem cell specification and organogenesis. *Plant Cell* **17**, 691-704.

Gross-Hardt, R., Lenhard, M. and Laux, T. (2002). WUSCHEL signaling functions in interregional communication during Arabidopsis ovule development. *Genes Dev.* **16**, 1129-1138.

Hempel, F. D., Zambryski, P. C. and Feldman, L. J. (1998). Photoinduction of flower identity in vegetatively biased primordia. *Plant Cell* **10**, 1663-1676.

Jeong, S., Trotochaud, A. E. and Clark, S. E. (1999). The Arabidopsis *CLAVATA2* gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *Plant Cell* **11**, 1925-1934.

Kayes, J. M. and Clark, S. E. (1998). *CLAVATA2*, a regulator of meristem and organ development in *Arabidopsis*. *Development* **125**, 3843-3851.

Laux, T., Mayer, K. F., Berger, J. and Jurgens, G. (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. *Development* **122**, 87-96.

Lenhard, M., Jurgens, G. and Laux, T. (2002). The *WUSCHEL* and *SHOOTMERISTEMLESS* genes fulfill complementary roles in *Arabidopsis* shoot meristem regulation. *Development* **129**, 3195-3206.

Li, J. and Nam, K. H. (2002). Regulation of brassinosteroid signaling by a GSK3/SHAGGY-like kinase. *Science* **295**, 1299-1301.

Mayer, K. F., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G. and Laux, T. (1998). Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. *Cell* **95**, 805-815.

Moore, I., Gälweiler, L., Grosskopf, D., Schell, J. and Palme, K. (1998). A transcription activation system for regulated gene expression in transgenic plants. *Proc. Natl. Acad. Sci. USA* **95**, 376-381.

Ni, J. and Clark, S. E. (2006). Evidence for functional conservation, sufficiency and proteolytic processes of the CLAVATA3 CLE domain. *Plant Physiol.* **140**, 726-733.

Olszewski, N. E., Martin, F. B. and Ausubel, F. M. (1988). Specialized binary vector for plant transformation: expression of the *Arabidopsis thaliana* AHAS gene in *Nicotiana tabacum*. *Nucleic Acids Res.* **16**, 10765-10782.

Otsuga, D., DeGuzman, B., Prigge, M. J., Drews, G. N. and Clark, S. E. (2001). *REVOLUTA* regulates meristem initiation at lateral positions. *Plant J.* **25**, 223-236.

Prigge, M. J., Otsuga, D., Alonso, J. M., Ecker, J. R., Drews, G. N. and Clark, S. E. (2005). Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in Arabidopsis development. *Plant Cell* **17**, 61-76.

Reddy, G. V. and Meyerowitz, E. M. (2005). Stem-cell homeostasis and growth dynamics can be uncoupled in the Arabidopsis shoot apex. *Science* **310**, 663-667.

Rojo, E., Sharma, V. K., Kovaleva, V., Raikhel, N. V. and Fletcher, J. C. (2002). CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signaling pathway. *Plant Cell* **14**, 969-977.

Running, M. P. and Meyerowitz, E. M. (1996). Mutations in the PERIANTHIA gene of Arabidopsis specifically alter floral organ number and initiation pattern. *Development* **122**, 1261-1269.

Running, M. P., Fletcher, J. C. and Meyerowitz, E. M. (1998). The WIGGUM gene is required for proper regulation of floral meristem size in Arabidopsis. *Development* **125**, 2545-2553.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K. F., Jurgens, G. and Laux, T. (2000). The stem cell population of *Arabidopsis* shoot meristems in maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. *Cell* **100**, 635-644.

Sessions, A., Weigel, D. and Yanofsky, M. F. (1999). The Arabidopsis thaliana MERISTEM LAYER 1 promoter specifies epidermal expression in meristems and young primordia. *Plant J.* **20**, 259-263.

Sharma, V. K., Ramirez, J. and Fletcher, J. C. (2003). The Arabidopsis CLV3-like (CLE) genes are expressed in diverse tissues and encode secreted proteins. *Plant Mol. Biol.* **51**, 415-425.

Shiu, S. H. and Bleecker, A. B. (2001). Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. *Proc. Natl. Acad. Sci. USA* **98**, 10763-10768.

Shpak, E. D., Berthiaume, C. T., Hill, E. J. and Torii, K. U. (2004). Synergistic interaction of three ERECTA-family receptor-like kinases controls *Arabidopsis* organ growth and flower development by promoting cell proliferation. *Development* **131**, 1491-1501.

Song, S. K. and Clark, S. E. (2005). POL and related phosphatases are dosage-sensitive regulators of meristem and organ development in Arabidopsis. *Dev. Biol.* **285**, 272-284.

DEVELOPMENT

Stone, J. M., Collinge, M. A., Smith, R. D., Horn, M. A. and Walker, J. C. (1994). Interaction of a protein phosphatase with an Arabidopsis serine/threonine receptor kinase. *Science* **266**, 793-795.

Stone, J. M., Trotochaud, A. E., Walker, J. C. and Clark, S. E. (1998). Control of meristem development by CLAVATA1 receptor kinase and kinase-associated protein phosphatase interactions. *Plant Physiol.* **117**, 1217-1225.

Turnbull, C. G., Booker, J. P. and Leyser, H. M. (2002). Micrografting techniques for testing long-distance signalling in Arabidopsis. *Plant J.* **32**, 255-262.

Wang, Z. Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T. et al. (2002). Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. *Dev. Cell* **2**, 505-513.

Williams, R. W., Wilson, J. M. and Meyerowitz, E. M. (1997). A possible role for kinase-associated protein phosphatase in the arabidopsis CLAVATA1 signaling pathway. *Proc. Natl. Acad. Sci. USA* **94**, 10467-10472.

Xiang, C., Han, P., Lutziger, I., Wang, K. and Oliver, D. J. (1999). A mini binary vector series for plant transformation. *Plant Mol. Biol.* **40**, 711-717.

Yin, Y., Wang, Z. Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T. and Chory, J. (2002). BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. *Cell* **109**, 181-191.

Yokoyama, R., Takahashi, T., Kato, A., Torii, K. U. and Komeda, Y. (1998). The Arabidopsis *ERECTA* gene is expressed in the shoot apical meristem and organ primordia. *Plant J.* **15**, 301-310.

Yu, L. P., Simon, E. J., Trotochaud, A. E. and Clark, S. E. (2000). *POLTERGEIST* functions to regulate meristem development downstream of the *CLAVATA* loci. *Development* **127**, 1661-1670.

Yu, L. P., Miller, A. K. and Clark, S. E. (2003). *POLTERGEIST* encodes a protein phosphatase 2C that regulates CLAVATA pathways controlling stem cell identity at *Arabidopsis* shoot and flower meristems. *Curr. Biol.* **13**, 179-188.

Zhao, J., Peng, P., Schmitz, R. J., Decker, A. D., Tax, F. E. and Li, J. (2002). Two putative BIN2 substrates are nuclear components of brassinosteroid signaling. *Plant Physiol.* **130**, 1221-1229.

Zhou, A., Wang, H., Walker, J. C. and Li, J. (2004). BRL1, a leucine-rich repeat receptor-like protein kinase, is functionally redundant with BRI1 in regulating *Arabidopsis* brassinosteroid signaling. *Plant J.* **40**, 399-409.

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