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POLTERGEIST Encodes a Protein Phosphatase 2C that Regulates CLAVATA Pathways Controlling Stem Cell Identity at *Arabidopsis* Shoot and Flower Meristems

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Summary

Background: Receptor kinases are a large gene family in plants and have more than 600 members in *Arabidopsis*. Receptor kinases in plants regulate a broad range of developmental processes, including steroid hormone perception, organ elongation, self-incompatibility, and abscission. Intracellular signaling components for receptor kinases in plants are largely unknown. The CLAVATA 1 (CLV1) receptor kinase in *Arabidopsis* regulates stem cell identity and differentiation through its repression of *WUSCHEL* (*WUS*) expression. Mutations at the *POLTERGEIST* (*POL*) gene were previously described as phenotypic suppressors of mutations within the *CLV1* gene. Genetic evidence placed POL as a downstream regulator of CLAVATA1 signaling.

Results: We provide evidence that *POL* functions in both the *CLV1-WUS* pathway and a novel *WUS*-independent *CLV1* pathway regulating stem cell identity. We demonstrate that *POL* encodes a protein phosphatase 2C (PP2C) with a predicted nuclear localization sequence, indicating that it has a role in signal transduction downstream of the CLV1 receptor. The N terminus of POL has a possible regulatory function, and the C terminus has PP2C-like phosphatase catalytic activity. Although the POL catalytic domain is conserved in other PP2Cs, the POL protein represents a unique subclass of plant PP2Cs. *POL* is broadly expressed throughout the plant.

Conclusions: POL represents a novel component of the CLV1 receptor kinase signaling pathway. The ubiquitous expression of *POL* and *pol* phenotypes outside the meristem suggest that POL may be a common regulator of many signaling pathways.

Introduction

Plants continuously form new organs by maintaining a population of undifferentiated stem cells throughout development. These cells are located at meristems. The shoot meristem gives rise to the entire aerial portion of the plant. The size and shape of the shoot meristem are defined early in embryogenesis and remain relatively constant during normal development. Shoot meristems maintain a tight balance between the proliferation of stem cells and the targeting of these cells toward differentiation. Several genes that are involved in the regulation of shoot meristem development have been identified.

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The *WUSCHEL (WUS)* gene, which encodes a homeodomain protein, is a key regulator of stem cell identity [1–3]. In the absence of *WUS*, stem cells are absent, and terminated shoots are formed. The repeated formation of terminated adventitious shoots governs post-embryonic development in *wus* mutant plants. Occasionally, floral primordia are formed, but flowers terminate before developing functional carpels. Misexpression of *WUS* is capable of specifying stem cells, especially in conjunction with another homeodomain protein, *SHOOT MERISTEMLESS* [4, 5].

CLAVATA genes (*CLV1*, *CLV2*, and *CLV3*) promote the progression of meristem stem cells toward differentiation through the repression of *WUS* expression. In wild-type plants, *WUS* expression is limited to a small population of cells in the center of the meristem underneath the three outermost cell layers, whereas *CLV1* expression overlaps and surrounds the *WUS* expression domain beneath the two outer cell layers [3, 6]. In *clv* mutant plants, the *WUS* expression area expands apically and laterally [3, 7]. Mutations in the *CLV* genes result in delayed organ initiation, leading to the accumulation of undifferentiated cells at the shoot and floral meristems and an increase in the overall size of these meristems. The enlarged floral meristems in *clv* plants, compared to wild-type, give rise to flowers with an increased number of floral organs and often form additional whorls of organs as well as undifferentiated cells within the gynoecea [8–10].

The *CLV* loci encode signal transduction components: CLV1, a receptor kinase [6]; CLV2, a receptor-like protein [11]; and CLV3, a small secreted protein that may act as a ligand for CLV1/CLV2 [12]. Although there have been studies of the biochemistry of CLV proteins at the plasma membrane [13–16], there is no understanding of the downstream cytoplasmic signaling factors.

POLTERGEIST represents an excellent candidate for a downstream CLV signaling component. Previous genetic analysis suggested that POL acts as a downstream negative regulator of CLV signaling and also revealed dominant interactions between *pol* and *wus* mutations [17]. Thus, further characterization of POL could greatly advance our understanding of downstream signaling factors in this key receptor system.

Here, we extend the genetic analysis of *POL* to reveal that *CLV* genes have additional signaling target(s) in addition to *WUS*. We show that *POL* encodes a functional protein phosphatase 2C (PP2C) with a putative N-terminal regulatory domain. Based on phylogenetic analyses, POL represents a new subclass of plant PP2Cs. *POL* mRNA is expressed in many tissues, suggesting that POL may function in regulating signal transduction in multiple developmental pathways.

Results

POL Functions in a *WUS*-Independent *CLV* Pathway

CLV loci and *WUS* function together to regulate the size of the shoot meristem. Several lines of evidence had

Table 1. *wus* Is Not Epistatic to *clv1* in a *pol* Mutant Background

Genotype	Average Number of Organs per Flower[a,b]		
	Sepals	Petals	Stamens[c]
wus-1	3.5 ± 0.1	3.5 ± 0.1	1.3 ± 0.1
clv1-4 wus-1	3.4 ± 0.1	3.3 ± 0.1	1.3 ± 0.2
pol-1 wus-1	2.9 ± 0.1	2.7 ± 0.2	0.6 ± 0.1
pol-1 clv1-4 wus-1	3.6 ± 0.1	3.4 ± 0.1	1.2 ± 0.1

[a] Values represent the mean number ± standard error of the mean of indicated organs.
[b] Fifty-four flowers were counted for each mean and standard error of the mean calculated.
[c] Whorl 3 organs that developed as filamentous organs were included in the count of stamens.

suggested that *WUS* was the sole target for CLV signaling. First, *wus* mutations are epistatic to *clv* mutations, as evidenced by the observations that *clv wus* flowers have the same number of floral organs as *wus* flowers [1, 3]. Second, *WUS* expression expands in *clv* mutants [3, 7]. Finally, the expressing *WUS* under control of the *CLV1* promoter can recreate the Clv1$^-$ phenotype [3].

We have previously reported that the *wus-1* mutation is incompletely dominant in a *pol-1* mutant background, which suggests that *POL* and *WUS* function in tight conjunction [17]. *pol* enhances the meristem defects of *wus* mutants, leading to both an overall reduction in post-embryonic organ formation and a reduction in floral organ number. This suggests the existence of a *WUS*-independent pathway regulating stem cell identity, contrary to previous evidence (see above).

To examine the relationship between the putative *WUS*-independent pathway and the *CLV* loci, we compared floral organ number in *wus-1*, *clv1-4*, *wus-1 clv1-4*, *wus-1 pol-1*, and *wus-1 clv1-4 pol-1* backgrounds (Table 1). We observed that whereas *wus-1* is epistatic to *clv1-4* in a *POL* wild-type background, consistent with previous findings [1, 3], *wus-1* is not epistatic to *clv1-4* in a *pol* mutant background. Thus, CLV1 is able to promote differentiation in a *wus-1 pol-1* background, yet it did not have this ability in a *wus-1* background. This finding implies the existence of an additional target(s) for CLV signaling.

Isolating the *POL* Gene

The genetic interactions of *POL* with other known regulators of *Arabidopsis* meristem development suggest that the POL protein may function downstream of the *CLV* loci as a negative regulator of signaling [17]. Thus, POL represents a possible unique intermediate between known cell-surface signaling components and WUS, one of the targets of CLV1 signaling. To further determine the role of POL, we isolated *POL* by linkage analysis.

We first determined the genetic linkage of *POL*. *pol-1 clv3-1* plants were out-crossed to mutant plants. In all cases, the frequency of *pol-1 clv3-1* plants in the F2 generation was higher than the 6.25% that would be expected if *POL* and *CLV3* were not linked. The recombination frequency in the F2 generation indicated that *POL* was 36 cM away from *CLV3* on chromosome 2. To determine the location of *POL* in relationship to *CLV3*, we crossed *pol-1 clv1-4* plants to *emb34* and *emb101-1* mutant plants that are genetically linked to the north and south of *CLV3*, respectively. Analysis of F2 plants indicated that *POL* is 13 cM south of *EMB101*.

For molecular linkage analysis, we utilized the sequence differences between Ler and Columbia ecotypes. The original line of *pol-1* contained a mixture of Ler and Columbia ecotypes, and the bottom arm of chromosome 2, where *pol-1* was located, was Columbia ecotype. Therefore, *pol-1 clv1-1* plants were crossed to *clv1-1* plants, which are Ler at the *POL* locus. DNA was collected from leaves of 477 F2 plants. The genotype of each F2 plant was verified in the F3 generation. Analysis of molecular markers indicated that *POL* was located between previously published CAPS markers GBF3 and 90J19T7. *POL* was located approximately 2.2 cM from GBF3 and 1.2 cM from 90J19T7. By sequencing randomly selected genes in this region and identifying polymorphisms between Ler and Columbia, we designed dCAPS markers (see Experimental Procedures) between the two ecotypes that assisted in isolating the location of *POL*. Detailed linkage analysis established that *POL* was located on bacterial artificial chromosome F14M4 in a region that consisted of six annotated genes in a region between dCAPS markers F14M4 and bHLH (Figure 1A). We determined that the gene corresponding with GenBank accession number AAC34239 was *POL* by sequencing the candidate gene from the mutant *pol* genomes (see below).

Sequencing of the *POL* cDNA from Ler inflorescences indicated that the database annotation of *POL* was incorrect. *POL* has a 2816 bp open reading frame that consists of three introns with lengths of 91 bp, 69 bp, and 85 bp and four exons (Figure 1B). Two expressed sequence tags (ESTs 247M5T7 and 158I21T7) that encoded part of the C terminus of POL were available in the public databases. Sequencing of the EST clones indicated that the 3′-UTR extends 102 bp beyond the predicted stop codon. In addition, database sequences of 5′-RACE products (APZL19d10F, RZL19e03F, SQ015h09F, SQ083b09F) and a full-length cDNA clone (R1651) indicated that the 5′-UTR begins 839 bp upstream of the predicted start codon and contains a 100 bp intron within the 5′-UTR (Figure 1B). The presence of multiple stop codons in the 5′-UTR suggests that the predicted open reading frame is the complete *POL* coding sequence.

POL Encodes a Predicted Nuclear-Localized Protein Phosphatase 2C

The *POL* open reading frame encodes an 856 amino acid protein predicted to contain protein phosphatase 2C (PP2C) motifs (Figure 1B) and a nuclear-localization motif. Similar to the overall structure of known plant PP2Cs, POL has a unique N terminus and a conserved C-terminal region that is predicted to have catalytic activity. The C-terminal portion of POL contains eleven conserved PP2C motifs that correspond to structural elements and functional residues [18–20]; however, the catalytic domain is interrupted by approximately 200 amino acids of novel sequence (Figure 1B). Comparing the primary sequence of POL with that of human PP2Cα,

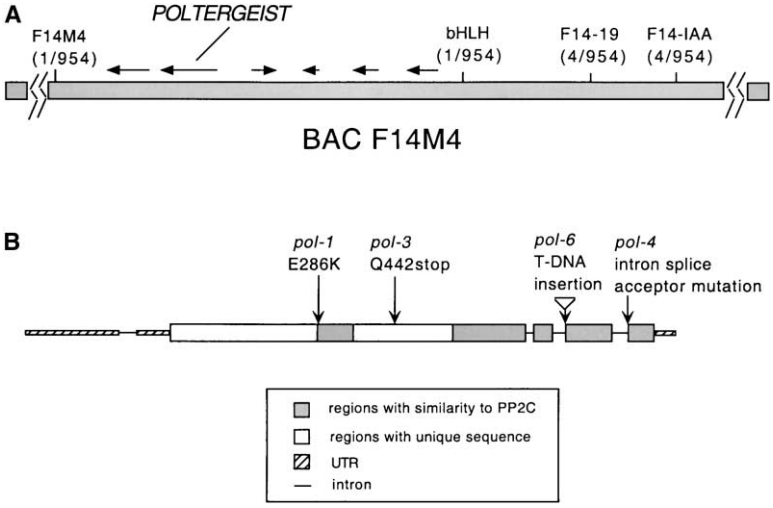

Figure 1. POL Cloning.

(A) Molecular mapping of *POL*. Individual F2 plants from a mapping population (see Experimental Procedures) were analyzed for meiotic recombination events via molecular markers that identified polymorphisms between Ler and Columbia ecotypes. The gene was delimited to a portion of BAC F14M4 between molecular markers F14M4 and bHLH. Values in parentheses represent the number of meiotic recombination events between each marker and the *pol* mutation. Horizontal arrows represent annotated genes [53] and their predicted direction of synthesis.

(B) Genomic structure of *POL*. The positions of the mutations found in *pol* alleles are indicated. Exons, introns, and 5′ and 3′ UTRs, as well as the PP2C-encoding domain, are indicated.

which has been crystalized [21, 22], we observed that POL contains the analogous amino acid residues (four aspartic acid, one glutamic acid, and one glycine) of human PP2Cα that interact with two Mg^{2+} or Mn^{2+} ions to form a binuclear metal center (Figure 2). The metal ions interact with six water molecules that function as nucleophiles and general acids in the dephosphorylation of the substrate [21, 22].

Mutations Disrupting the *POL* Gene

The *POL* gene was amplified and sequenced from genomic DNA of homozygous *pol* mutants. *pol-1*, *pol-3*, and *pol-4* [23] each have a single base pair substitution typical of ethylmethane sulfonate-induced mutations, whereas *pol-6* is disrupted by a T-DNA insertion (Figure 1B). Based on the average number of carpels formed in *pol clv1-1* mutant flowers, we classified the *pol-1* allele as an intermediate suppressor and the *pol-3* and *pol-4* alleles as weak suppressors of the $Clv1^{-}$ phenotype [17]. The mutations in the two weak *pol* alleles appear to encode truncated proteins. In *pol-3*, a nonsense mutation results in a translational stop after 442 amino acids, eliminating the bulk of the conserved phosphatase domain. In *pol-4*, the intron acceptor site of intron 2 is changed from AG to AA, which is predicted to result in incorrect splicing of the pre-mRNA and probably the loss of at least one-third of the wild-type protein, including portions of the conserved PP2C domain. In the intermediate *pol-1* allele, a conserved glutamic acid that is involved in metal binding in human PP2Cα is changed to lysine by a missense mutation (Figures 1B and 2; [21, 22]).

POL Has PP2C Activity

POL has motifs that are conserved in other PP2Cs (Figure 1B) and metal binding sites that are involved in phosphatase activity in humans and bacteria (Figure 2; [19, 24]). To determine whether POL is functional as a phosphatase, we performed in vitro phosphatase assays. The POL PP2C-like domain was expressed in *E. coli* as a maltose binding protein (MBP) fusion protein, MBP-POL(CAT) (see Experimental Procedures). This construct excluded the first 233 amino acid residues of the N terminus. MBP and MBP-POL(CAT) proteins were expressed in *E. coli* and affinity purified (Figure 3A). The isolated proteins were then tested for the ability to dephosphorylate ^{32}P-casein, an artificial substrate commonly used in assaying PP2C activity [25, 26].

MBP-POL(CAT) dephosphorylated ^{32}P-casein in the presence of Mg^{2+} and Mn^{2+}, whereas MBP did not (Figure 3B). Compared to the MBP background, MBP-POL

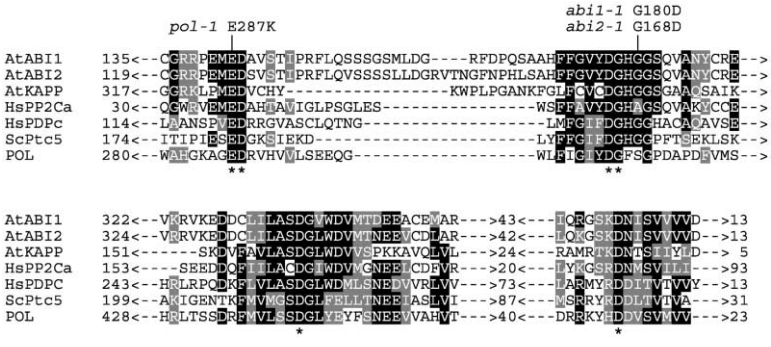

Figure 2. Metal-Interacting Acidic Residues Are Conserved among *Arabidopsis*, Human, and *S. cerevisiae* PP2Cs

Shown are sequence alignments of amino acids that are predicted to bind Mg^{2+} or Mn^{2+}. Domains were selected based on similarity to metal cofactor binding domains in human PP2Cα [21, 22]. Sequences used in this analysis were as followed: ABI1 from *Arabidopsis* (At4g26080), ABI2 from *Arabidopsis* (At5g57050), KAPP from *Arabidopsis* (At5g19280), PP2Calpha from human (AAB21784), PDP-2 catalytic subunit from human (Q9P2J9), Ptc5 from *S. cerevisiae* (NP_014733), and POL from *Arabidopsis*. The number of residues in front of and between the domains is indicated. Conserved residues are shaded in black. Conservative changes are shaded in gray. Dashes represent gaps that were introduced to optimize the alignments. Asterisks represent residues known to interact with metal cofactors in human PP2Calpha [22]. Amino acids that are changed in *pol-1*, *abi1-1*, and *abi2-1* missense mutants are indicated.

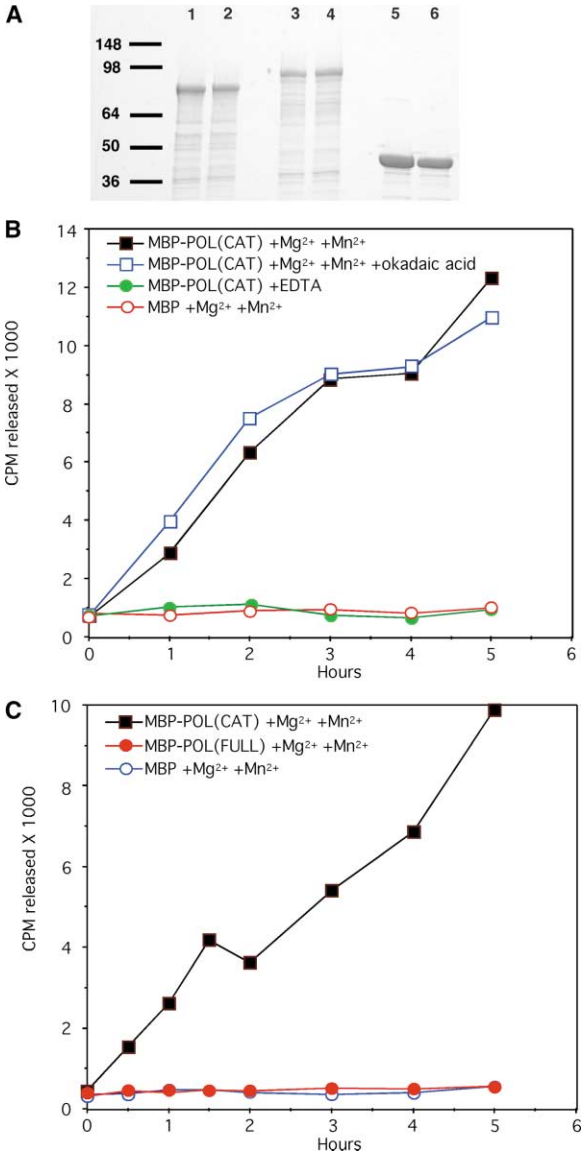

Figure 3. POL Has PP2C Catalytic Activity

(A) MBP-POL(CAT) (lanes 1 and 2), MBP-POL(FULL) (lanes 3 and 4), and MBP (lanes 5 and 6) were purified from in BL21 *E. coli* cells.

(B) MBP-POL(CAT) and MBP were placed in phosphatase reactions with ^{32}P-casein in the presence of 10 mM Mg^{2+} and Mn^{2+}, 10 mM Mg^{2+} and Mn^{2+} plus 10 μM okadaic acid, or 10 mM EDTA (see Experimental Procedures). At specified time points, released ^{32}P was isolated from ^{32}P-casein by phenol-chloroform separation. Protein phosphatase activity is represented by the amount of CPM (counts per minute) released.

(C) MBP-POL(FULL), MBP-POL(CAT), and MBP were placed in phosphatase reactions with ^{32}P-casein in the presence of 10 mM Mg^{2+} and Mn^{2+}.

(CAT) exhibited stable phosphatase activity over a 5 hr period. To verify that, like other PP2Cs, POL(CAT) requires Mg^{2+} and Mn^{2+} for phosphatase activity, we conducted MBP-POL(CAT) phosphatase reactions in the absence of Mg^{2+} and Mn^{2+} and in the presence of EDTA, which would chelate contaminating dications. Under these conditions, phosphatase activity was undetectable and equivalent to that in the MBP control (Figure 3B). Another characteristic of PP2Cs is their insensitivity to okadaic acid. MBP-POL(CAT) in the presence of Mg^{2+} and Mn^{2+} displayed similar rates of dephosphorylation of ^{32}P-casein in the presence and absence of okadaic acid, which inhibits the activity of other classes of phosphatatses (Figure 3B). Based on the ability of MBP-POL(CAT) to dephosphorylate ^{32}P-casein in the presence of Mg^{2+} and Mn^{2+} and its insensitivity to okadaic acid, POL is a functional PP2C.

The N Terminus of POL Functions as a Putative Regulatory Domain

The N-terminal region of POL does not have similarity to proteins with known functions. To determine if the unique region has regulatory function, we performed in vitro phosphatase assays with ^{32}P-casein as the substrate [25, 26]. Recombinant proteins that consisted MBP fused to full-length POL, MBP-POL(FULL), were expressed in *E. coli* and affinity purified (Figure 3A). The extracted proteins were then tested for phosphatase activity in the presence of Mg^{2+} and Mn^{2+}.

Interestingly, MBP-POL(FULL) had no detectable phosphatase activity and was equivalent to the MBP negative control (Figure 3C). Compared to MBP-POL(FULL) and MBP, MBP-POL(CAT) maintained stable phosphatase activity in the simultaneous reactions. These results suggest that the POL N-terminal domain inhibits POL phosphatase activity and may play a regulatory function in vivo.

POL Is Broadly Expressed within the Plant and the Meristem

The genetic studies of *pol* with other developmental regulators had indicated that POL might act in multiple plant organs [17]. To identify where POL may function, we performed RT-PCR on RNA isolated from wild-type Ler inflorescences, stage 13–16 flowers, stems, cauline leaves, rosette leaves, and roots by using *ROC1*-, *WUS*-, and *POL*-specific primers. As expected, *ROC1* was detected in all tissues (Figure 4A) [27], which indicated that RNA was present in the samples collected. *POL* mRNA was detected in all tissues tested (Figure 4A).

Because RT-PCR analysis indicated that *POL* was expressed in many tissues, we sought to determine the pattern of POL expression at the inflorescence. The meristem regulators *CLV1*, *CLV3*, *WUS*, and *STM* are all expressed within specific subdomains of the shoot meristem in patterns that in many ways reflect their functions and interactions [2, 6, 12, 28, 29]. The first 2168 nucleotides of the *POL* open reading frame showed poor nucleotide sequence similarity to other *Arabidopsis* sequences. ^{35}S-labeled antisense and sense riboprobes corresponding to this region were generated and hybridized to sections of wild-type Ler inflorescence tissue.

In the shoot meristem, *POL* was expressed throughout the meristem (Figure 4B). Based on longitudinal sections, *POL* was expressed in all cell layers. *POL* signal persisted on the flanks of the shoot meristem where new floral primordia were formed (Figure 4B). Sense controls suggested that the signal was specific for *POL* and not due to high background.

In the floral meristem, *POL* expression continued

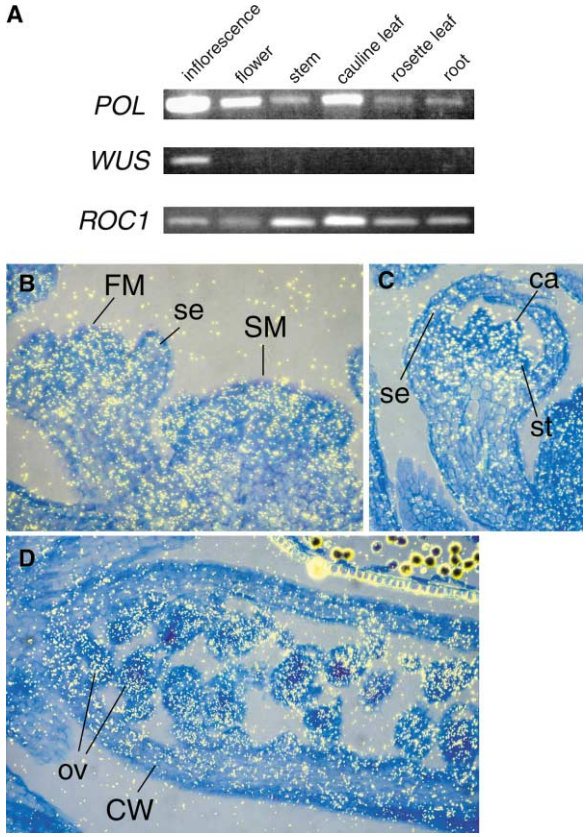

Figure 4. *POL* Is Expressed in All the Tissues Tested

(A) Total RNA was isolated from wild-type inflorescences, stage 13–16 flowers, pedicels, stems, cauline leaves, rosette leaves, and roots. RNA was reverse transcribed and amplified with *POL*-, *WUS*-, and *ROC*-specific primers.

(B–E) Wild-type inflorescences were probed with a *POL* antisense riboprobe. *POL* is expressed throughout the shoot meristem (SM) and flower meristem (FM), as well as young primordia such as sepals (se), stamens (st) and carpels (ca). During gynoecium development, very little *POL* expression is detected in carpel walls (CW), but it is detected at high levels in developing ovules (ov). Sense controls revealed that this signal is specific for *POL* (our unpublished data).

throughout development. As flower organs were initiated and established, *POL* was expressed in all four whorls (Figures 4B and 4C). As the floral organs matured, *POL* expression decreased. By stage 12 of flower development [30], *POL* expression was limited to the ovule (Figure 4D).

POL mRNA was also detected in young pedicels but was later absent in mature organs (our unpublished data). Furthermore, *POL* was expressed within the stem in the outer cell layers but was absent from the region presumed to contain the pith (Figure 4B).

POL Represents a Novel Class of PP2C Proteins that Is Conserved in *O. sativia*

PP2C proteins have been identified in many organisms. One suite of characteristics of PP2Cs is eleven conserved protein motifs and amino acid residues that can bind to Mg^{2+} or Mn^{2+} [18–22]. The metal ions interact with six water molecules that function in phosphatase catalysis. The POL catalytic domain has all of these properties and was found to be similar to many sequences with known functions in plants, animals, and yeast (Figure 2). However, POL BLAST searches indicated that, beyond the conserved catalytic domains, the protein does not have high similarity to other known plant PP2Cs. Furthermore, the POL catalytic domain has structures that are unique as compared to known plant PP2Cs.

To identify other genes that are phylogenetically related to POL, we used the POL catalytic domain to perform BLAST searches. Several *Arabidopsis* (AtPLL) and *O. sativia* (rice; OsPOL and OsPLL) protein sequences with unknown functions were identified. To determine the phylogenetic relationships between these proteins and POL, we performed a similarity analysis with the predicted catalytic domains of the proteins by using a heuristic tree-building program. Because POL had the least protein similarity to At3g16560, At3g55050, and At3g12620 of the BLAST-identified proteins, these proteins were defined as outgroups. In 10,000 bootstrap replicates, 97% of replicates placed POL in the same clade as AtPLL1 (AAC3686; see Experimental Procedures), AtPLL2 (NP195860), AtPLL3 (NP187551), AtPLL4 (AAL38775), AtPLL5 (AAK32783), OsPOL, and OsPLL (Figure 5; see Experimental Procedures). AtPLL2/AtPLL3 and AtPLL4/AtPLL5 appear to represent the most recent duplications within this clade. AtPOL, AtPLLs, OsPOL, and OsPLL all share an insertion in the same region of the catalytic domain. This insertion appears to be unique within this subclass of plant PP2Cs. Of the bootstrap replicates, 84% placed POL in the same subclade as OsPOL (Figure 5). When the predicted catalytic domain sequences were compared, it was found that POL was 98% similar to and 83% identical to OsPOL, which supports the placement of *Arabidopsis* POL in the same clade as *O. sativia* POL. These results suggest that the duplications within this family occurred prior to the divergence of the two species.

Discussion

Models of POL Regulation of Stem Cell Identity

Previous evidence was consistent with WUS being the sole target of CLV1 signaling. *wus* mutants are fully epistatic to *clv1* mutants, and *WUS* overexpression under control of the *CLV1* promoter recreated the Clv1$^-$ phenotype. Our analyses of *pol clv wus* triple mutants and *pol wus* double mutants revealed that in a *pol* background, CLV1 can function in the absence of WUS. POL therefore functions in both the *WUS*-dependent CLV pathway and a novel *WUS*-independent CLV pathway regulating meristem development. Because the *clv wus* flowers do not have significantly more flowers than *wus* flowers, we propose that POL normally inhibits the WUS-independent CLV pathway (Figure 6). Thus, it is only in the absence of POL and WUS that the independent pathway is detectable. We propose two models in which POL regulates the pathways promoting stem cell identity: (1) POL functions as a negative regulator of CLV signaling in both the WUS-dependent and the WUS-

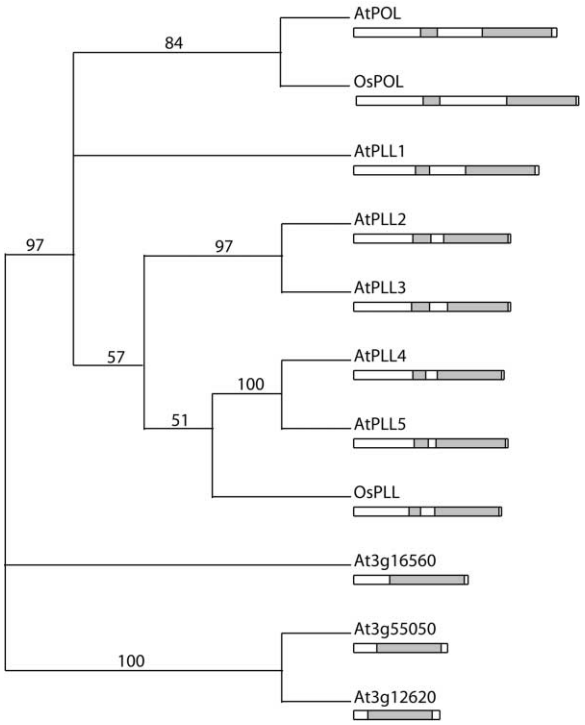

Figure 5. Phylogenetic Analysis between Plant PP2C Catalytic Domains

For determining phylogenetic relationships, protein sequences of the conserved PP2C domains for putative plant proteins related to POL were aligned and then used in ClustalX. Ten thousand bootstrap replicates were performed. At3g16560, At3g55050, and At3g12620 were defined as outgroups. Sequences used in this analysis were as follows: POL, AtPLL1 (AAC3686), AtPLL2 (NP195860), AtPLL3 (NP187551), AtPLL4 (AAL38775), AtPLL5 (AAK32783), At3g16560, At3g55050, and At3g12620 from *Arabidopsis*; and OsPOL and OsPLL from *O. sativia* (see Experimental Procedures). Relative protein structure is indicated below each gene name. White bars represent unique domains, and gray bars represent PP2C-like domains.

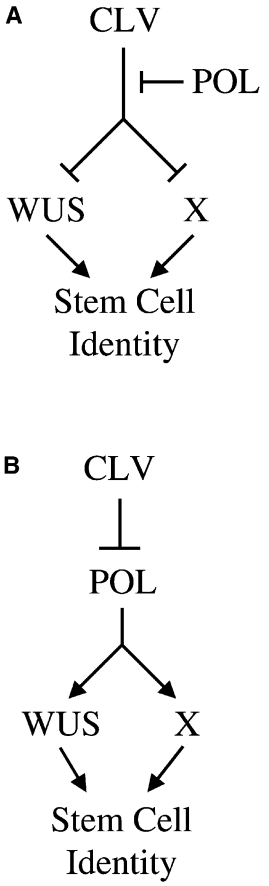

Figure 6. Models of POL Function in the Stem Cell Identity Pathways

(A) POL is a negative regulator of CLV regulation of WUS and an unknown factor X, both of which promote stem cell identity.

(B) POL is a positive regulator of WUS and an unknown X factor and is inactivated by CLV signaling.

independent CLV pathways; or (2) POL is a positive signaling component of the targets of both CLV pathways and is inactivated by CLV signaling (Figure 6).

The first model would suggest that POL acts analogously to the phosphatase KAPP, but at a different point in the signaling pathway. KAPP contains a forkhead-associated domain (FHA) that acts to mediate binding to phosphorylated CLV1 and a type 2C protein phosphatase domain [13, 14, 31]. KAPP is capable of dephosphorylating CLV1, and it is a negative regulator of CLV1 signaling at the plasma membrane. POL similarly contains a novel regulatory domain at the N terminus, as well as a C-terminal phosphatase domain. Thus, by analogy to KAPP, POL would bind a phosphorylated intermediate of CLV1 signaling and inactivate the component by reversing phosphorylation.

In the second model, POL would activate the downstream targets of CLV signaling (WUS and an unknown factor). CLV signaling would regulate WUS through inactivation of POL. Our observation that POL is a protein phosphatase would suggest the presence of an additional kinase that would constitutively phosphorylate the targets of POL. The model would further require the presence of factors that have functional overlap with POL in the activation of WUS and the other unknown target because *pol* mutations are not epistatic to *clv* mutations.

The two models equivalently explain our genetic results and cannot be distinguished at this time. More studies of this pathway at the biochemical level and identification of other genes involved in shoot meristem development are required to determine if either model is correct.

POL PP2C Domain Structure

The POL protein possesses the eleven conserved PP2C domains. The POL C-terminal domain, which encompassed the conserved PP2C domains, was capable of dephosphorylating ^{32}P-casein. After a period of 5 hr, MBP-POL(CAT) dephosphorylated 6% of the ^{32}P-casein substrate (Figure 3B). Although this domain had stable phosphatase function, the level of dephosphorylation was lower than the level for *Arabidopsis* KAPP under the same conditions [26]. The difference between POL and KAPP phosphatase activity in these in vitro experiments may be due to the fact that the enzymes were

expressed in *E. coli*, to the use of an artificial substrate casein, and/or to plant cofactors missing from the reaction.

A comparison of POL to other plant PP2Cs, such as KAPP, suggests that POL activity may also be affected by its the unique structure, which differs from that of all other previously described plant PP2Cs. The eleven conserved phosphatase domains in POL are interrupted by a unique sequence of more than 200 amino acids (Figure 1B). Domains I, II, and III, which contain one metal binding site, are separated from domains IV through XI, which contain the second metal binding site. The approximately 200 amino acids that interrupt the PP2C domain in POL have no similarity to any protein with known function based on BLAST searches (our unpublished data). Based on our phylogenetic analyses, the insertion in plants is a derived trait that is unique to the POL clade.

Although the insertion in POL appears to be a derived trait within the small POL clade, several other PP2Cs interestingly have insertions at similar positions. Bovine Mg^{2+}-dependent Ca^{2+}-inhibited protein phosphatase (MCPP; GenBank accession number AAB39357) is a PP2C with a large internal region between domains III and IV that is highly acidic. Phosphatase analyses showed that although the MCPP has broad substrate specificity, it has a preference for basic proteins [32]. This preference suggests that the acidic internal domain may be involved in substrate specificity by electrostatic interactions. The insertion is also present in PP2Cs from a variety of organisms, such as rice OsPOL, *S. cerevisiae* Ptc1 and Ptc5 [33], human PP2Cγ [34, 35] and pyruvate dehydrogenase phosphatase [36], and mouse FIN13 [37]. The repeated location of the insertion in many species, which do not share a common ancestral origin of the insertion based on phylogeny, suggests that this trait may have evolved independently many times. It is possible that the internal domain may have a similar function in all the organisms as a regulator of substrate specificity in these proteins. Based on consensus sequence analysis, the POL internal domain is predicted to be composed of two α-helices and three random coil domains (our unpublished data).

pol-1 May Function as a Dominant-Negative Form of POL

POL mutant alleles partially suppress the *CLV* mutant phenotypes. *pol-1* is semi-dominant in the *clv* mutant background. Plants homozygous for *pol-1 clv1-1* have fewer floral organs than *pol-1/POL clv1-1* plants, which in turn have fewer organs than *clv1-1* homozygous plants [17]. *pol-1* also suppresses the organ development pathway regulated by *CLV2* in an incompletely dominant manner. In the *clv2* background, plants heterozygous for *pol-1* have shorter pedicels than homozygous *POL* plants and longer pedicels than homozygous *pol-1* plants. Furthermore, *pol-1* has a more severe phenotype than the putative null alleles *pol-3* and *pol-4*. Sequencing of the *pol-1* allele indicated that it has a missense mutation causing a glutamic acid (Glu) to become a lysine (Lys). The Glu288 residue of pol-1 corresponds to Glu37 that is involved in metal cofactor binding in human PP2Cα [21, 22]. This residue is conserved among known PP2Cs from bacteria to humans and is critical for phosphatase activity [19, 24].

The pol-1 protein has a dramatic change from a negatively charged Glu288 to a positively charged lysine residue, which presumably alters the function of the site. Because *pol-1* encodes a full-length protein with presumed reduced catalytic activity, pol-1 protein may act in a dominant-negative fashion. *abi1-1* and *abi2-1* missense mutations of a glycine to aspartic acid near a metal binding site result in proteins that may function as dominant negatives (Figure 2; [38–41]). In *pol-1/POL* plants, the dominant-negative pol-1 protein may bind to the presumed phosphorylated target and lock it in a permanent phosphorylated state because of the lack of catalytic activity.

In contrast to *pol-1*, the *pol-3* and *pol-4* mutations may be null alleles; they are predicted to result in the loss of more than one-third of the wild-type POL, including the majority of the conserved PP2C domain. Because of the weak suppression of $Clv1^{-}$ phenotypes by *pol-3* and *pol-4*, we suspect that the catalytic activity of POL may be functionally replaced by the activity of another protein(s) in the *pol-3* and *pol-4* mutant backgrounds. The protein(s) most likely to exhibit functional overlap with POL would be those with similar sequence and structure. Based on the phylogenetic analysis of POL (Figure 5), PLL1 is a possible candidate. PLL1 has 67% identity and 83% similarity to the POL catalytic domain. Similar to POL, PLL1 also has an approximately 200 amino acid insertion (Figure 5) between the conserved PP2C domains III and IV. All of the AtPLL genes have insertions within the same region, although the insertion varies in size. In addition, all AtPLL proteins also share additional regions of sequence similarity with the N terminus of POL (our unpublished data). Our phylogenetic analysis of the *POL* gene family is consistent with a recent comprehensive *Arabidopsis* phosphatase alignment [42]; however, Kerk and coworkers did not include POL and PLL1 in their list of *Arabidopsis* protein phosphatases.

Conclusions

The genetic interactions of *POL* with other known regulators of *Arabidopsis* meristem development suggested that the POL protein functions downstream of the *CLV* loci as a negative regulator of signaling. Further analyses indicated that POL and WUS may function in close association with each other [17]. We report that *POL* encodes a functional PP2C that has a predicted nuclear localization signal and belongs to a unique a subclass of plant PP2Cs. The N terminus of POL potentially provides a negative regulatory function for catalytic activity, and the C terminus has protein phosphatase catalytic activity. Because of the ubiquitous expression of *POL* mRNA and its mutant phenotypes, we propose that POL regulates multiple features of plant development through the regulation of receptor kinase signaling. Based on our genetic analyses of *pol clv wus* plants, *POL* regulates not only the *CLV-WUS* pathway but also a *WUS*-independent *CLV* pathway regulating stem cell identity.

POL adds to the limited number of known and putative

receptor kinase signaling intermediates, including the GSK3-like kinase BIN2, BZR1 and BES1, which participate in brassinosteroid signaling [43–45]; the PP2C KAPP, which interacts with several receptor kinases [13, 14, 26, 46]; and the arm-repeat protein ACR1, which acts in *Brassica* self-incompatibility [47, 48]. Identifying such intermediates is a key to dissecting the signaling pathways and the mechanism of signaling.

Experimental Procedures

Plant Growth

Seeds were sown on a 1:1:1 mix of soil:perlite:vermiculite with approximately 1 g/pot of Osmocote 14-14-14 and imbibed at 4°C for 7 days. Plants were grown at 22°C under approximately 800 footcandles of constant cool white fluorescent light.

Molecular Mapping

Leaf samples from individual F2 plants of a mapping population were collected, and genotypes of F2 plants were verified in the F3 generation. Genomic DNA was isolated as described [49].

Individual DNA samples were tested for linkage with published CAPS, SSLPs [50–52], or novel dCAPS (available upon request) designed based on comparison of the Ler sequence to the published *Arabidopsis* Columbia genome [53]. Polymerase chain reactions (PCR) were composed of 10% DNA sample, 1× Qiagen PCR Buffer, additional 1.25 mM $MgCl_2$ (total $MgCl_2$ concentration of 3.75 mM), 2 mM dNTP, 2.5 ng/μl of each primer, and 2 units of Qiagen Taq polymerase. PCR cycles consisted of 94°C disassociation, 53°C annealing, and 72°C extension for 30 s to 1.5 min repeated 30 times. CAPS and dCAPS were digested with appropriate restriction enzymes. DNA bands were resolved on 2%–3% agarose gels.

Candidate genes were amplified by PCR with appropriate primers and DNA from Ler, *pol-1*, *pol-3* and *pol-4* plants, and then the isolated PCR products were sequenced.

Generation of Recombinant POL Protein

POL cDNA from Ler inflorescences was PCR amplified with primers that flanked the entire *POL* coding sequence (5′-GGATCCAAAATG GGAAACGGGACT-3′ and 5′-TAAGGATCCTTATCTATTGAATTTTT GTT-3′) and primer sets that flanked the *POL* phosphatase-encoding domain sequence (5′-GGATCCTCTAGACGATCTGGAGGG TTA-3′ and 5′-CTGCAGTTATCTATTGAATTTTTGTT-3′), which were respectively called POL(FULL) and POL(CAT). PCR products were purified and subcloned into pCRII (Invitrogen) and sequenced. Correct gene fragments were subcloned in frame with the MBP coding sequence in pMALc2 (New England Biolabs). pMALc2-POL(FULL), pMALc2-POL(CAT), and pMALc2 were transformed into BL21 *E. coli* cells.

E. coli cultures were induced with 0.1 mM IPTG at 37°C for 4 hr. Recombinant proteins were isolated as described [14]. Proteins were eluted with buffer containing 50 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 10 mM maltose, 10% glycerol, and 200 mM NaCl. Samples were quantitated with BioRad Protein Assay according to the manufacturer's instructions and visualized on Biorad 4%–15% Tris-Cl gradient gels stained with GelCode Blue (Pierce).

Phosphatase Assay

Casein was radiolabeled with ^{32}P as described [25]. Unincorporated γ-ATP was separated from the ^{32}P-casein by gel filtration on a PD-10 column (Amersham Pharmacia) equilibrated with 50 mM Tris-Cl (pH 7.0), 0.1 mM EGTA, and 5% glycerol. SDS-PAGE was performed on each fraction with Biorad 4%–15% gradient Tris-Cl gel. Staining protein gels with GelCode Blue (Pierce) determined protein quantity, and exposing them to X-ray film identified fractions containing ^{32}P-casein.

Phosphatase activity was determined in reactions containing 10 μg recombinant protein, approximately 3.5×10^6 CPM ^{32}P-casein, 0.2 μg/μl BSA, 50 mM Tris-Cl (pH 7.0), 0.1 mM EGTA, 1.8 mM maltose, 36.48 mM NaCl, 2.5% glycerol, and either 10 mM each Mg^{2+} and Mn^{2+} with and without 10 μM okadaic acid or 10 mM EDTA at room temperature [25, 26]. Aliquots of 10% of the original reaction were removed at each time point and treated with phenol-chloroform. Radioactivity in the aqueous layer was measured in a liquid scintillation counter.

Reverse Transcription PCR

Inflorescence, flower, stem, cauline leaf, and rosette leaf tissue samples were collected from Ler plants grown as described above. Root tissue was collected from Ler seedlings grown on plates with 4.2 g/liter Murashige and Skoog salts, 0.3% sucrose, and 8.0 g/liter agar. Plates were placed vertically under constant cool white fluorescent light after being imbibed at 4°C for 2 days. Other tissues were collected from plants grown as described (see above), with the flower sample isolated from open stage 13–16 flowers [30].

For RNA isolation, tissue samples in liquid nitrogen were macerated with a mortar and pestle. Tissue was mixed with 1 ml Trizol (Gibco BRL) and incubated at room temperature for 5 min. 0.2 ml chloroform was then added, and samples were incubated at room temperature for 2 min. Samples were centrifuged at 14000 RPM at 4°C for 15 min. The aqueous RNA layer was removed, and RNA was precipitated with 0.25 ml 1.2 M sodium citrate, 0.8 M NaCl, and 0.25 ml isopropanol. Samples were incubated at room temperature for 10 min and then centrifuged at 14,000 RPM at 4°C for 10 min. RNA pellets were washed with 75% ethanol and then dissolved with 0.1% DEPC-treated H_2O. RNA was quantified with a spectrophotometer.

Reverse transcription was performed on 1 μg of isolated RNA with the Thermoscript RT-PCR System (Gibco BRL) according to the manufacturer's instructions. cDNA was amplified with the following primer sets: for *ROC1*, 5′-GATCTACGGGAGCAGTTCG-3′ and 5′-TTCTCGATGGCCTTTACCAC-3′ [27]; for *WUS*, 5′-CCCGGGG ATGGAGCCGCCACAGCATCAG-3′ and 5′-AGCGTACGTCGATGTT CCAGATAAGCATCG-3′ [2]; and for *POL*, 5′-AGTGCTCTTGATGG GATTCCG-3′ and 5′-GAGAAATCCAGCACCGAAAGC-3′. PCR reactions were composed of 2% cDNA, 1× Qiagen PCR Buffer, additional 1.5 mM $MgCl_2$ (total $MgCl_2$ concentration of 4.0 mM), 2 mM dNTP, 4.0 ng/μl of each primer, and 5 units of Qiagen Taq polymerase. PCR conditions consisted of 94°C disassociation, 53°C annealing, and 72°C extension for 30 s to 1.0 min repeated 30 times. PCR products were resolved by gel electrophoresis on 1% agarose gel.

In Situ Hybridization

Tissue samples were collected from Ler plants after being placed in the growth chamber for 30 days. *POL* cDNA from Ler inflorescences was PCR amplified with primers that flanked the entire *POL* sequence (5′-GGATCCAAAATGGGAAACGGGACT-3′ and 5′-TAA GGATCCTTATCTATTGAATTTTTGTT-3′). PCR product was subcloned into pCRII (Invitrogen) and sequenced. The clone was digested with BamHI and HindIII and subcloned into pGEM7zf. Tissue fixation, riboprobe, and in situ procedure were conducted as described [6] with the exception that the riboprobe was not hydrolyzed.

Alignments and Phylogeny

Protein sequences were aligned with the ClustalW program and then manually adjusted. Protein alignments were presented in BOXSHADE format and modified in Microsoft Word.

BLAST searches identified proteins with high similarity to POL. The GenBank accession numbers for *PLL2*, *PLL3*, *PLL4*, and *PLL5* are NP195860, NP187551, AAL38775, and AAK32783, respectively. Because the annotation of *PLL1* (AAC36186) omitted the last exon, the remainder of the amino acid sequence was deduced by manually splicing the 3′-end of the genomic sequence. The GenBank accession numbers for the *O. sativia* genomic DNA sequence used for phylogenetic analysis are AC087220 and AC104433 for *OsPOL* and *OsPLL*, respectively. For deducing amino acid sequences, genomic sequences were spliced with the NetGene2 prediction program (http://www.cbs.dtu.dk).

For generating phylogenetic trees, catalytic domains were determined based on consensus sequence and aligned with the ClustalX program [54]. Entering alignments in PAUP4 then determined parsimony. Ten thousand bootstrap replicates were performed.

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