PLANT SCIENCES

Functional innovations of PIN auxin transporters mark crucial evolutionary transitions during rise of flowering plants

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Flowering plants display the highest diversity among plant species and have notably shaped terrestrial landscapes. Nonetheless, the evolutionary origin of their unprecedented morphological complexity remains largely an enigma. Here, we show that the coevolution of cis-regulatory and coding regions of *PIN-FORMED* (*PIN*) auxin transporters confined their expression to certain cell types and directed their subcellular localization to particular cell sides, which together enabled dynamic auxin gradients across tissues critical to the complex architecture of flowering plants. Extensive intraspecies and interspecies genetic complementation experiments with *PIN*s from green alga up to flowering plant lineages showed that *PIN* genes underwent three subsequent, critical evolutionary innovations and thus acquired a triple function to regulate the development of three essential components of the flowering plant *Arabidopsis*: shoot/root, inflorescence, and floral organ. Our work highlights the critical role of functional innovations within the *PIN* gene family as essential prerequisites for the origin of flowering plants.

INTRODUCTION

To adapt to new and challenging environments, large evolutionary transitions from anatomically simple green algae to developmentally complex multicellular land plants (e.g., flowering plants) took place during plant diversification (*1*). These transitions were driven by crucial innovations in the anatomy of plants (*2*). Flowering plants (angiosperms), the latest emerging land plant lineage with highly complex morphological structures, appeared around 135 million years ago and evolved from nonflowering seed plants according to fossil evidence from the Cretaceous period (*3*). Flowering plants are currently the most highly diverse plant group; with around 350,000 species, they make up 90% of all living land plant species (*3*). Recently, scientists have speculated that their rapid and noteworthy success was underpinned by a series of architectural innovations. One of the most important among these innovations is the formation of floral organs, which allows these plants to access new reproduction possibilities and thus facilitates a faster propagation (*3*, *4*).

Almost all aspects of development and patterning of flowering plants are mediated by local auxin concentration/gradients, which rely on directional cell-to-cell polar auxin transport (PAT) (*5*–*8*). The auxin efflux carriers PIN-FORMED (PIN) proteins, localized at the plasma membranes (PMs) and restricted to a particular side of the cell (*9*–*12*), are the most crucial components of PAT (*13*–*16*) that control many aspects of flowering plant patterning and adaptive growth. In the model flowering plant *Arabidopsis thaliana*, the null mutant *pin-formed1* (*pin1*) shows severe defects with a naked inflorescence meristem, which does not initiate flowers (*17*). Mutants in other members of the family have multiple developmental defects including those in embryogenesis (*18*), organogenesis (*6*), root and shoot tropisms (*6*), apical hook formation (*19*), stomata patterning (*20*), and many others (*14*).

Given a variety of PIN roles in patterning and development of the flowering plant *Arabidopsis*, tracing the diversification and functional evolution of the *PIN* family members will help us to understand how the functional innovations of *PIN*s along plant evolution endow flowering plants with a high complexity of tissues compared to their predecessors. In addition, such work will also enable us to understand the origin of flowering plants from an evolutionary perspective at the molecular level.

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RESULTS

Genetic analysis of diverse PIN functions in *Arabidopsis* patterning

In general, in terms of the PIN phylogenetic tree (*21*), there are two types of PINs in the plant kingdom: noncanonical PINs and canonical PINs (fig. S1). Unlike noncanonical PINs, which are localized predominantly to the endoplasmic reticulum (ER), the canonical PINs are predominantly targeted to the PM and mediate the intercellular PAT to regulate plant development and patterning (*21*, *22*). In model flowering plant *A. thaliana*, five of the eight PIN members belong to canonical PINs, distributed in three disparate PIN clades: PIN1 clade (AtPIN1), PIN2 clade (AtPIN2), and PIN3 clade (i.e., AtPIN3, AtPIN4, and AtPIN7) (Fig. 1A).

Each of these three PIN clades showed its specific function in plant patterning and growth. As previously reported, the loss-of-function *Arabidopsis pin1* single mutant displayed severe defects in the development of shoot with naked inflorescence and no floral organ formation (Fig. 1B) (*17*), while *pin2* mutant showed defective root gravitropism (fig. S2D) (*23*). The triple mutant *pin3/4/7* had a shoot with more branches (*24*) and roots with impaired gravitropism (Fig. 1B and fig. S2E) (*25*). Next, to understand the functional relationship between PIN clades, we combined every pair out of three PIN clades to construct higher-order *Arabidopsis pin* mutants for phenotypic analysis. Unlike the phenotype of *pin1* and *pin3/4/7* mutants, a quadruple mutant *pin1/3/4/7* showed severe defects in both shoot and root development (Fig. 1B and fig. S2, G and I) (*26*). However, the shoot of the *pin1/2* double mutant displayed a similar phenotype to that of the *pin1* mutant, while its root phenotype resembles the *pin2* mutant (fig. S2F) (*5*). Similarly, the shoot of the quadruple mutant *pin2/3/4/7* had a phenotype like the triple

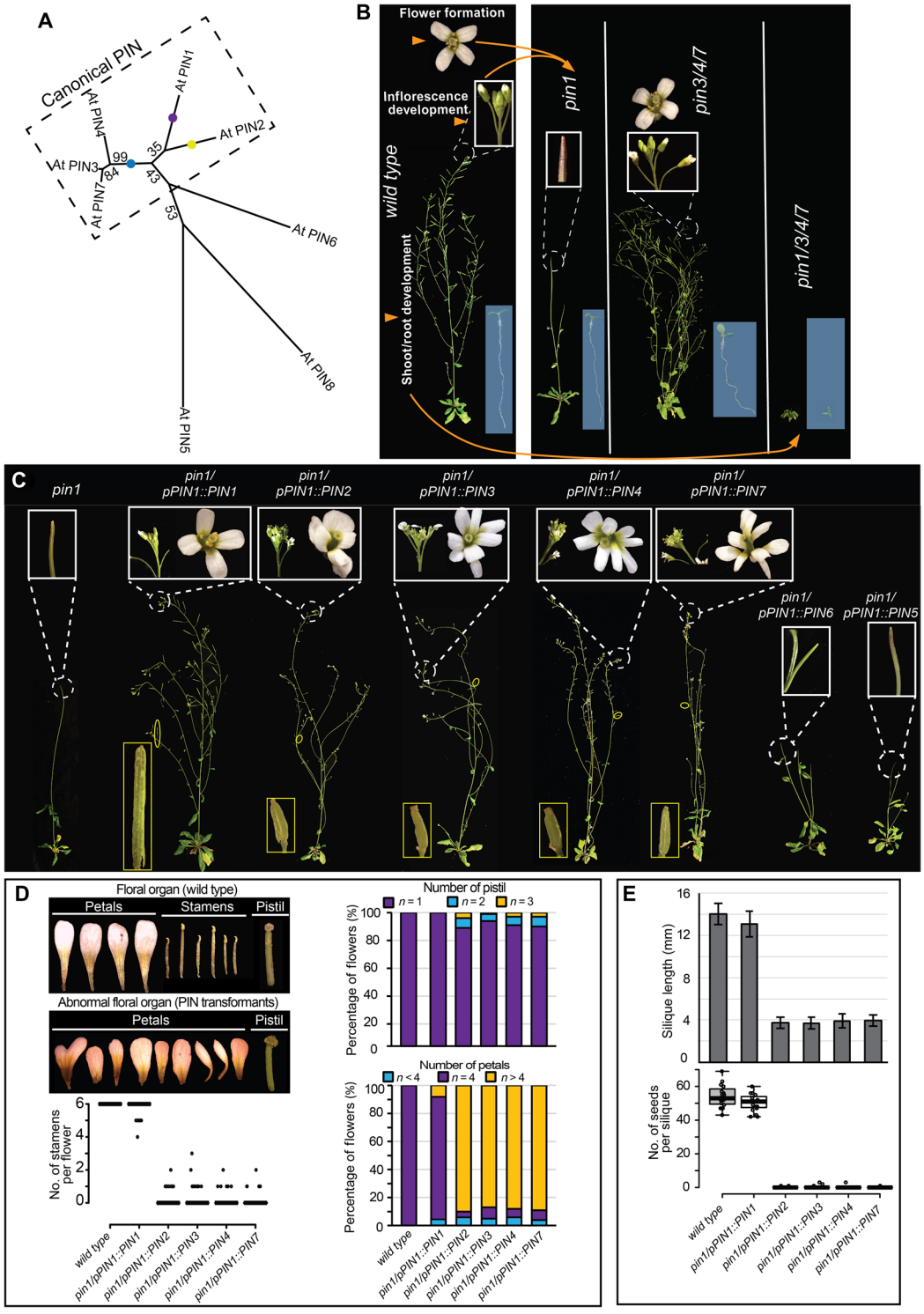

Fig. 1. Intraspecies genetic complementation analysis of *Arabidopsis* PIN function in its inflorescence/floral organ patterning. (**A**) Phylogenetic relationship of the homologous *PIN* members in the flowering plant *A. thaliana*. The canonical PINs are divided into three clades: PIN1, PIN2, and PIN3/4/7. (**B**) Phenotypical analysis of the *Arabidopsis pin* mutants with disruption of one or two clades of the canonical PINs (PIN1 and PIN3/4/7). Phenotypical analysis of the shoots with 7-week-old plants and the roots with 1-week-old seedlings. (**C**) The *Arabidopsis* null mutant *pin1* showed severe defects in inflorescence/floral organ formation. Genetic complementation experiments with *Arabidopsis* paralogous PINs showing that of the *A. thaliana* canonical *PIN*s (*PIN1*, *PIN2*, *PIN3*, *PIN4*, and *PIN7*) and noncanonical *PIN*s (*PIN5* and *PIN6*); all canonical PINs, but not noncanonical PINs, were able to complement the naked inflorescence of *pin1* mutant. Only *PIN1* was able to fully rescue the defective floral organ with no stamens and structurally aberrant petals. Phenotypic analysis of the shoot system with 7-week-old plants. (**D**) Anatomical structure of flower from the PIN transformants in (C). $n = 100$ flowers from each transgenic line. (**E**) Silique length and seed number per silique of PIN transformants in (C). $n = 13$ to 19 siliques from each transgenic line. Photo credit: Yuzhou Zhang, Institute of Science and Technology (IST) Austria.

mutant *pin3/4/7*, and its root showed gravitropic defects like the *pin2* mutant (fig. S2H) (*27*).

These results indicate that the *Arabidopsis* PIN1 and PIN3 clades regulate the *Arabidopsis* developmental patterning (fig. S2J). Specifically, we showed PIN1 exclusive functions in *Arabidopsis* (i) inflorescence development and (ii) floral organ formation, as well as its redundant function in (iii) shoot/root development together with PIN3/4/7 members.

Specific functions of PIN homologous in the development of different *Arabidopsis* tissues

To test the capacity of *Arabidopsis* PIN members in floral organ patterning, we separately introduced different *Arabidopsis* paralogous PIN members into *Arabidopsis pin1* mutant under the control of a 3.2-kb *Arabidopsis PIN1* promoter.

The results showed that of these canonical *Arabidopsis* PIN members, only PIN1 was able to fully rescue the *pin1* defects in the floral organ, which is composed of four petals, six stamens, and one pistil in wild-type *Arabidopsis* (Fig. 1, C and D). In contrast, all other canonical PINs (AtPIN2, AtPIN3, AtPIN4, and AtPIN7) failed to replace AtPIN1 function in *Arabidopsis* flower development and thus resulted in a significantly reduced number of stamens (in most cases no stamens), an occasionally increased number of pistils ($n \geq 1$), and abnormally shaped and often increased number of petals (Fig. 1, C and D, and fig. S3). These defective floral organs were sterile and did not produce any viable seeds in the siliques (Fig. 1E and fig. S4). Together, these results confirmed the exclusive function of AtPIN1 to regulate the development of an intact and fertile floral organ in *Arabidopsis*.

The same genetic complementation experiments showed that all five canonical AtPIN proteins (PIN1/PIN2/PIN3/PIN4/PIN7), but not the noncanonical AtPINs (PIN5 and PIN6), can rescue the defective *pin1* mutant with naked inflorescence (Fig. 1C). This suggests that these five *Arabidopsis* canonical PINs share the common capacity in maintaining *Arabidopsis* inflorescence development.

To determine which of the *Arabidopsis* PINs evolved the capacity in its shoot/root patterning (Fig. 1B), driven by *AtPIN1* promoter, we expressed the *Arabidopsis PIN* paralogs into its *pin1/3/4/7* quadruple mutant. The genetic complementation showed that noncanonical AtPINs (PIN5/PIN6) failed to replace PIN1 function, and thereby, all seedlings of the transformants displayed the defective shoot/root development (table S1), similar to the dwarf phenotype of the *Arabidopsis pin1/3/4/7* mutant (Fig. 2A). In contrast, all five *Arabidopsis* canonical PINs showed their capability to rescue the severe shoot/root defects in *pin1/3/4/7* mutant (e.g., PIN2 84.7% $n = 72$, PIN3 85.1% $n = 67$, and PIN4 77.6% $n = 49$, respectively) (Fig. 2A and table S1). This indicated that, in addition to the inflorescence development, all *Arabidopsis* canonical PINs had the same ability to regulate *Arabidopsis* shoot/root formation.

Together, all five canonical PINs have comparable capacities to regulate shoot/root formation and inflorescence development of *Arabidopsis*. However, the capacity to regulate its floral organ formation is PIN1 specific.

The capacity of PIN homologs to establish auxin gradient in *Arabidopsis* root tissue

Tissue/organ formation depends on the local auxin gradients, which are recognized as the pattern- and organ-organizing signal (*5*, *10*). Thus, we used the *Arabidopsis* root as a tractable tissue to investigate whether canonical PINs enable plant developmental patterning by facilitating the establishment of auxin gradient (maxima). To monitor localized auxin response maxima in the root (*10*, *28*), we introduced the synthetic auxin-responsive reporter *DR5rev::GFP* (green fluorescent protein) into these *Arabidopsis* transgenic lines. Unlike the wild type, barely any DR5 signal was detected in the *pin1/3/4/7* mutant root tip, indicating aborted auxin maximum formation (Fig. 2, B and C). Genetic complementation assays showed that all five *Arabidopsis* canonical PIN proteins were capable of restoring the aborted auxin maximum of *pin1/3/4/7* mutant, which is indicated by their comparable *DR5rev::GFP* intensities in the root tip of these transgenic lines (Fig. 2, D to H and L, and fig. S5). However, the noncanonical PINs (PIN5 and PIN6) failed to reestablish the auxin maximum in the root tip of the *pin1/3/4/7* mutant, as the DR5 signal remained nearly undetectable (Fig. 2, I and J). These results suggested that all five *Arabidopsis* canonical PINs have a comparable capacity to mediate auxin maximum formation in the *Arabidopsis* root tip, consistent with their equivalent function in *Arabidopsis* root development (Fig. 2, A and K).

Evolutionary divergence of cis-regulatory and coding domains of *PIN* family members

Genetic complementation assays indicated that all five *Arabidopsis* canonical PINs shared the equivalent capacity to regulate inflorescence and shoot/root developments (Figs. 1C and 2A), whereas the genetic assays with *pin* mutants suggested that only AtPIN1 regulated the inflorescence development and only AtPIN1/3/4/7 regulate the shoot/root development (Fig. 1B and fig. S2, B to H). The above inconsistency suggests that the five canonical *AtPIN*s with a comparable capacity to regulate inflorescence and shoot/root developments are partly selected to control *Arabidopsis* development as a result of their differentiated expression patterns (Fig. 3A). Here, we used the *Arabidopsis* root as a model to study the contribution of expression pattern difference of PIN members in plant development. As previously mentioned, AtPIN1 is expressed in the central part of the root cylinder (stele cells) and shows a basal subcellular localization to orient auxin flow toward the root tip (Fig. 3, B and C) (*5*, *10*, *29*), while AtPIN2 is expressed specifically at the outer sides of the root and shows apical localization to orient auxin flow away from the root tip (Fig. 3D) (*7*). However, in the *pin1;pPIN1::PIN2* transgenic line we constructed, when PIN2 was ectopically expressed in the stele cells under the control of *Arabidopsis PIN1* promoter (Fig. 3E), it was localized to the basal side of the stele cells to direct auxin flow towards the root tip like PIN1 protein does (Fig. 3, C and E). The result was in concordance with our observations that PIN2 is functionally equivalent to PIN1 in *Arabidopsis* root development when driven by *AtPIN1* promoter (Fig. 2A). Together, these findings indicate that, in addition to the exclusive function of AtPIN2 in root gravitropism (*30*), PIN2 also has the capacity in the regulation of root patterning comparable to the other four *Arabidopsis* canonical PINs (AtPIN1/3/4/7) (Fig. 2A). However, this ability of AtPIN2 was hidden and taken over by the other *Arabidopsis* canonical PINs because of the divergence of expression patterns of these *PIN* members.

In addition to the evolutionary divergence of cis-regulatory elements, the coding region of *PIN* members has also undergone evolutionary changes, indicated by genetic complementation results that *PIN1*, but not *PIN2*, was capable of regulating *Arabidopsis* flower development when driven under the *AtPIN1* promoter (Fig. 1C). The PIN proteins are defined by a conserved modular protein structure

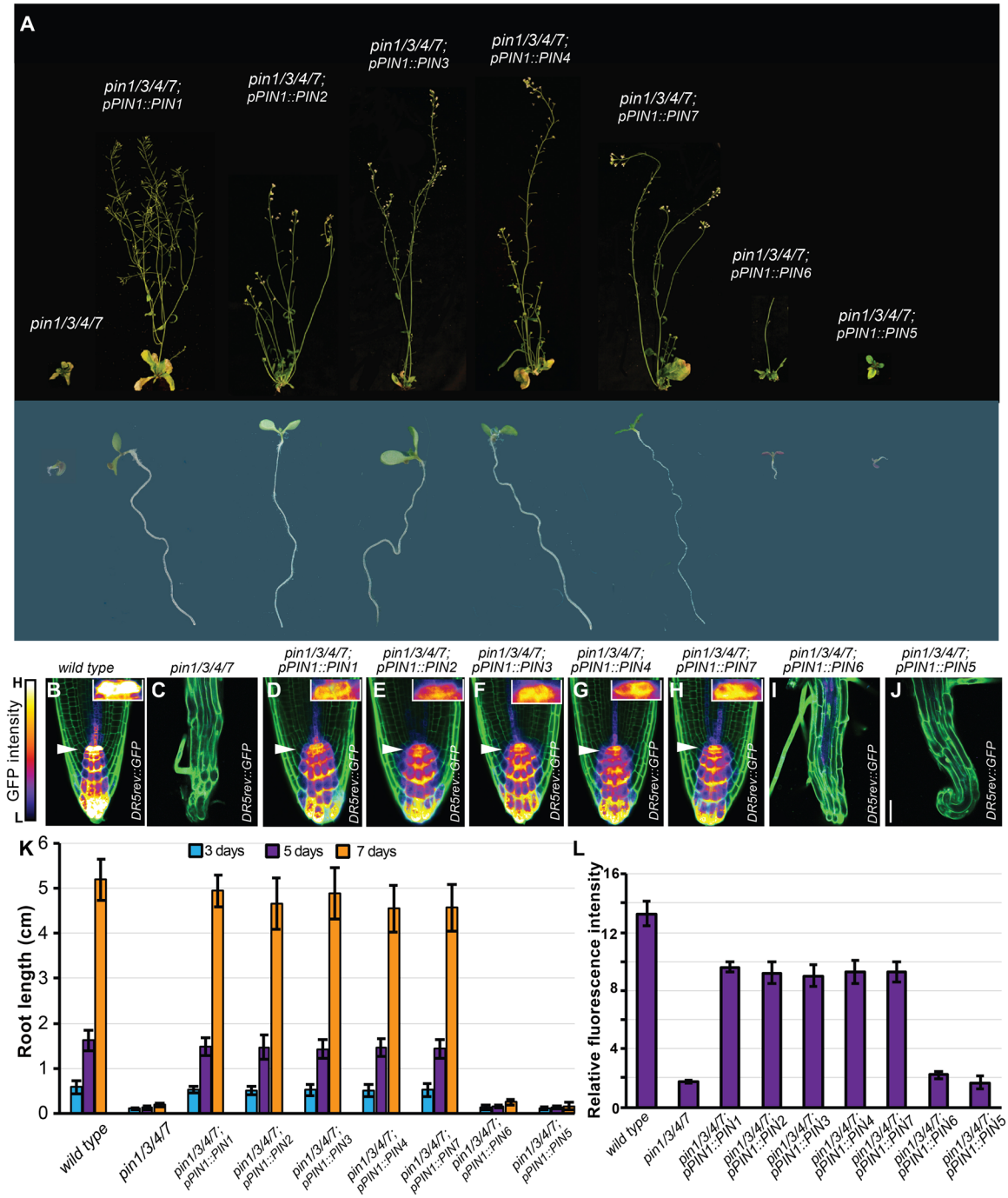

Fig. 2. Intraspecies genetic complementation analysis of *Arabidopsis* homologous PIN function in its shoot/root patterning. (**A**) The *Arabidopsis* loss-of-function *pin1/3/4/7* quadruple mutant showed severe defects with the arrested shoot and root development, and genetic complementation experiments with *Arabidopsis* canonical *PINs* (*PIN1*, *PIN2*, *PIN3*, *PIN4*, and *PIN7*) and noncanonical *PINs* (*PIN5* and *PIN6*) showed that only the canonical PINs were able to rescue the severe shoot/root defects of *Arabidopsis pin1/3/4/7* quadruple mutant. Phenotypic analysis of the shoots with 7-week-old plants and the roots with 1-week-old seedlings. (**B** to **J**) Auxin maximum in root tips of the PIN transformants in (A), indicated by the synthetic auxin-responsive reporter *DR5rev::GFP* in 7-day-old *Arabidopsis* seedlings. The level of *DR5rev::GFP* expression is reflected by signal intensity. The GFP channel images are shown in pseudocolor, and the intensity scale is shown at the left (H, high; L, low). The white arrowhead indicates the quiescent center. Scale bar, 20 μm. (**K**) Root lengths of wild type, *pin1/3/4/7* mutant, and PIN transformants after growing for of 3, 5, and 7 days, respectively. Data represent means ± SD ($n = 11$ roots from each line). (**L**) Quantification of the *DR5rev::GFP* signal intensity in wild type, *pin1/3/4/7* mutant, and PIN transgenic lines shown in (D) to (J). Data represent means ± SD ($n = 6$ roots from each line). Photo credit: Yuzhou Zhang, Institute of Science and Technology (IST) Austria.

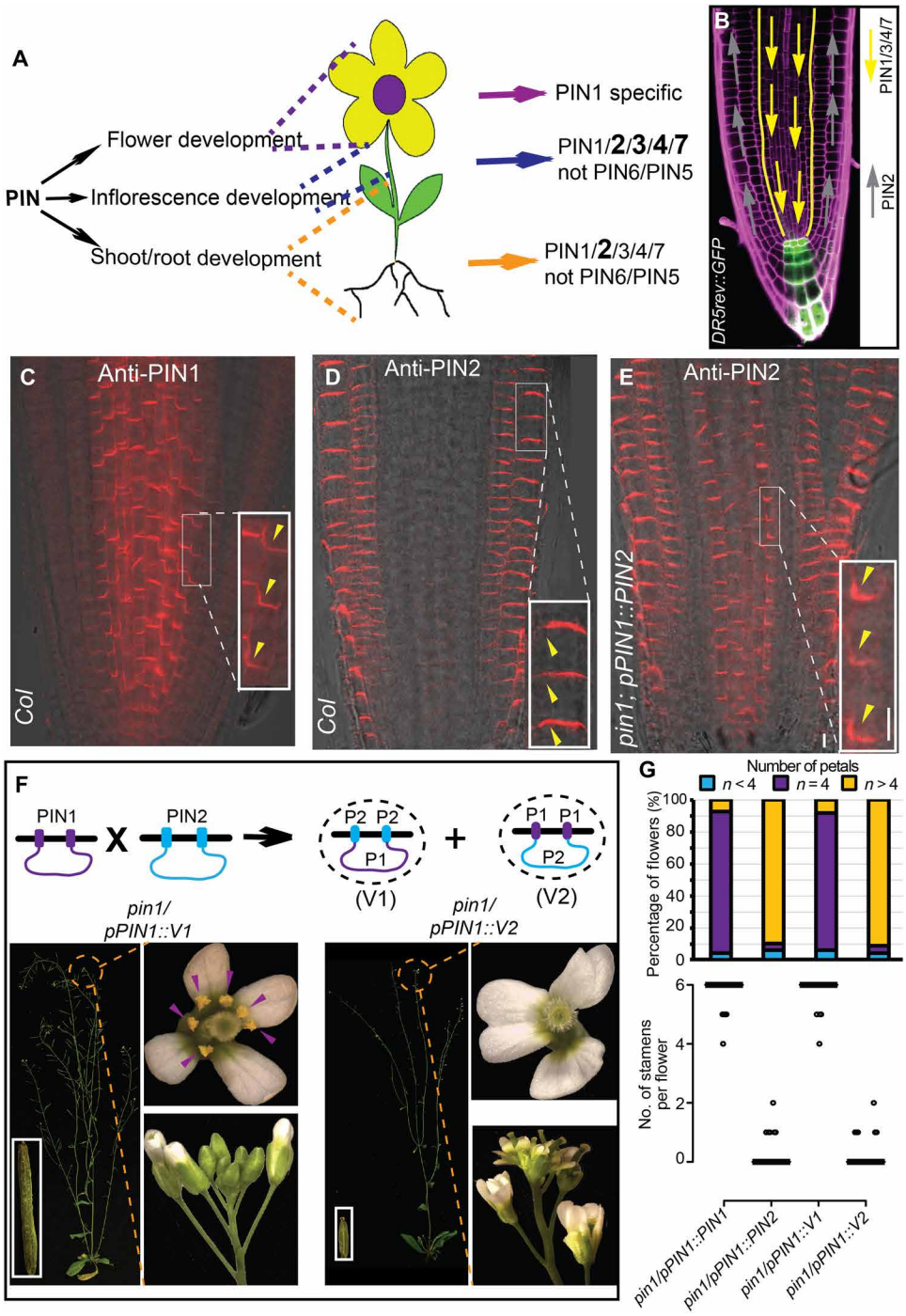

Fig. 3. The divergence of both coding and cis-regulatory domains of *Arabidopsis PIN* members. (**A**) Schematic diagram showing three functions of *Arabidopsis* canonical *PIN*s (*PIN1*, *PIN2*, *PIN3*, *PIN4*, and *PIN7*) and noncanonical *PIN*s (*PIN5* and *PIN6*) in plant patterning according to the evolutionarily functional analysis. (**B**) Schematic diagram of the generation of an auxin maximum in the root by the *Arabidopsis* PIN auxin transporters. (**C** and **D**) Immunolocalization showing that PIN1 is specifically expressed in the stele of *Arabidopsis* root with rootward (basal) subcellular localization (C), while PIN2 is exclusively expressed in the lateral sides of *Arabidopsis* root with shootward (apical) subcellular localization in epidermal cells (D). (**E**) In *pin1;pPIN1::PIN2* transgenic lines, immunolocalization showing that the ectopic expression of AtPIN2 in the stele (middle of the root) under the control of *PIN1* promoter results in its basal subcellular localization similar to that of AtPIN1 (C). The yellow arrowheads indicate the PIN polarity in root cells. Scale bar, 10 μm. (**F**) Function of chimeric PIN proteins (V1 and V2) with domains swapped between PIN1 and PIN2 in floral organ development. (**G**) Statistical analysis of the flower patterns of the transgenic lines carrying the chimeric PIN proteins V1 and V2 in (F). The transgenic lines with PIN1 or PIN2 expressed in *Arabidopsis pin1* mutant background under the control of *PIN1* promoter were used as the control. $n = 100$ flowers from each transgenic line. Photo credit: Yuzhou Zhang, Institute of Science and Technology (IST) Austria.

encompassing N- and C-terminal transmembrane domains (TMDs) and a central, cytosolic hydrophilic loop (HL) (*31*). To find out which part of the PIN protein contributes to its divergent function, we performed intragenic domain swapping experiments between AtPIN1 and AtPIN2 (Fig. 3F). When both the N- and C-TMDs of the AtPIN1 were replaced with N- and C-TMDs of AtPIN2, this chimeric PIN (V1) still showed the ability to restore the flower defects of *Arabidopsis pin1* mutant. However, the chimera V2 with HL of AtPIN1 replaced by that of AtPIN2 failed to maintain floral organ development, indicated by that the *pin1/pPIN1::V2* transgenic line produced an infertile flower with very few stamens and other defective floral organ phenotypes, resembling that of transgenic line *pin1/pPIN1::PIN2* with few viable seeds in its siliques (Fig. 3, F and G, and fig. S6). These results suggested that the HL domain, rather than the TMDs of *Arabidopsis* canonical PIN, experienced strong evolutionary selection, which contributed to their functional divergence.

The phosphorylation of the PIN protein plays a crucial role in determining PIN activities and localization (*14*, *32*). On the basis of the previously published work (*33*–*35*), we summarized the differences in the phosphorylation sites of HL domains between AtPIN1 and AtPIN2 (fig. S7). We proposed that the phosphosite difference of HL might contribute to the divergent function of AtPIN1 and AtPIN2 in *Arabidopsis* floral organ development. This is further supported by the fact that Ser^{337}, a phosphosite important for AtPIN1 function in flower development, is missing in the PIN2-HL (*34*).

In summary, the evolutionary changes of both the coding and the cis-regulatory regions of *AtPIN* genes determine their differentiated functions in *Arabidopsis* patterning.

PIN function for *Arabidopsis* shoot/root development originated in land plants

All the canonical AtPINs have the capability to regulate the development of *Arabidopsis* shoot/root (Fig. 2A). Next, we addressed the functional origin of *PIN* genes that regulate *Arabidopsis* shoot/root development. To this end, we performed interspecies genetic complementation experiments by introducing orthologous PINs, from green alga up to flowering plant lineages, into the *Arabidopsis pin1/3/4/7* quadruple mutant under the control of *PIN1* promoter. The results showed that the green algal *Klebsormidium flaccidum KfPIN*, a PIN member showing lower sequence similarity of HL to that of canonical PIN in land plants (*36*), was unable to rescue the severe shoot/root defects of *Arabidopsis pin1/3/4/7* mutant (0% $n = 57$; Fig. 4A). However, the canonical PINs (*MpPINZ* and *PpPINA*) from the living representatives of the early diverging land plant *Marchantia polymorpha* and *Physcomitrella patens* had partly acquired the competence to replace the PIN1/3/4/7 function (67.7% $n = 62$ and 57.1% $n = 49$, respectively), and therefore, they substantially complemented the defective shoot/root development of *Arabidopsis pin1/3/4/7* mutant (Fig. 4, B and C, and table S2). This suggested that PIN with the function to regulate *Arabidopsis* shoot/root patterning might have evolved in land plants after their separation from the green algal lineage. Furthermore, the canonical PINs from other advanced land plant lineages, i.e., the lycophyte *Selaginella moellendorffii* (*SmPINR*), the gymnosperm *Pinus taeda* (*PtPINE* and *PtPINH*), and flowering plants *Amborella trichopoda* (*AmtPIN1a*), *A. thaliana* (*AtPIN1*), and *Capsella rubella* (*CarPIN1*), all showed the capacity to replace the AtPIN1 function to rescue the *pin1/3/4/7* defects in *Arabidopsis* shoot/root development (Fig. 4, D to I, and table S2). These results indicate that only the canonical PIN proteins from land plants are capable of regulating the shoot/root patterning of the flowering plant *Arabidopsis*.

Land plant canonical PINs mediate *Arabidopsis* root-tip auxin maximum formation

To examine the functional differences of PINs from different plant species in establishing the *Arabidopsis* root-tip auxin maximum, we introduced auxin-responsive reporter *DR5rev::GFP* into these *PIN* transformants. The results showed that the green algal *KfPIN* failed to restore the aborted auxin maximum in the root tip of *pin1/3/4/7* mutant (Fig. 4, J and K, and fig. S8), whereas the *MpPINZ* and *PpPINA* from nonvascular land plants could partially reestablish the auxin maximum in *pin1/3/4/7* mutant root tip (Fig. 4, L and M). Furthermore, the canonical PINs (*SmPINR*, *PtPINH*, *PtPINE*, *AmtPIN1a*, *AtPIN1*, and *CarPIN1*) from all the representative vascular plants successfully restored the defective auxin maximum in *pin1/3/4/7* mutant root tip (Fig. 4, N to S and U). These results were consistent with phenotypical observations that the canonical PINs from land plants could complement the defective root development of *Arabidopsis pin1/3/4/7* mutant (Fig. 4T).

PIN function for *Arabidopsis* inflorescence development originated in vascular plants

All the *Arabidopsis* canonical PINs have evolved the ability to regulate the development of inflorescence, another crucial component besides shoot/root for flowering plants. However, the functional origin of *PIN* genes that maintain *Arabidopsis* inflorescence during plant evolution is still unclear. To address this question, similarly, we performed interspecies complementation experiments by introducing orthologous PINs, from green alga up to flowering plant lineages, into the *Arabidopsis pin1* mutant under the control of *AtPIN1* promoter. The experiments revealed that the green alga *KfPIN* was unable to rescue the *Arabidopsis pin1* defects in the inflorescence development (Fig. 5A). Similarly, both canonical PINs from the nonvascular land plant liverwort (*MpPINZ*) and moss (*PpPINA*) failed to complement the null mutant *pin1* naked inflorescence (Fig. 5, B and C). In contrast, the *PIN* from basal vascular plant lycophyte (*SmPINR*) was capable of rescuing the naked inflorescence phenotype of *pin1* mutant (Fig. 5D). These results suggest that the canonical PIN capable of regulating *Arabidopsis* inflorescence development might have emerged in the basal vascular plant lineage.

Moreover, the canonical PINs from the basal seed plant gymnosperm (*PtPINH* and *PtPINE*) and the flowering plants (*AmtPIN1a*, *AtPIN1*, *and CarPIN1*) had the competence to restore the naked inflorescence of *Arabidopsis pin1* mutant (Fig. 5, E to I). This implies that the function of canonical *PIN* genes to regulated *Arabidopsis* inflorescence development might be widely present in vascular plant lineages, including seed plants and flowering plants.

PIN function for *Arabidopsis* flower development originated in flowering plants

Floral organs represent the third essential component of flowering plants. To investigate the functional origin of the *PIN* genes that regulate *Arabidopsis* flower patterning during plant evolutionary history, we used the interspecies genetic experiments by expressing these *PIN1* orthologous genes into the *Arabidopsis* null mutant *pin1* to test their function in floral organ development. Both the canonical PINs from the basal vascular plant lycophyte *S. moellendorffii* (*SmPINR*) and the basal seed plant *P. taeda* (*PtPINE* and *PtPINH*)

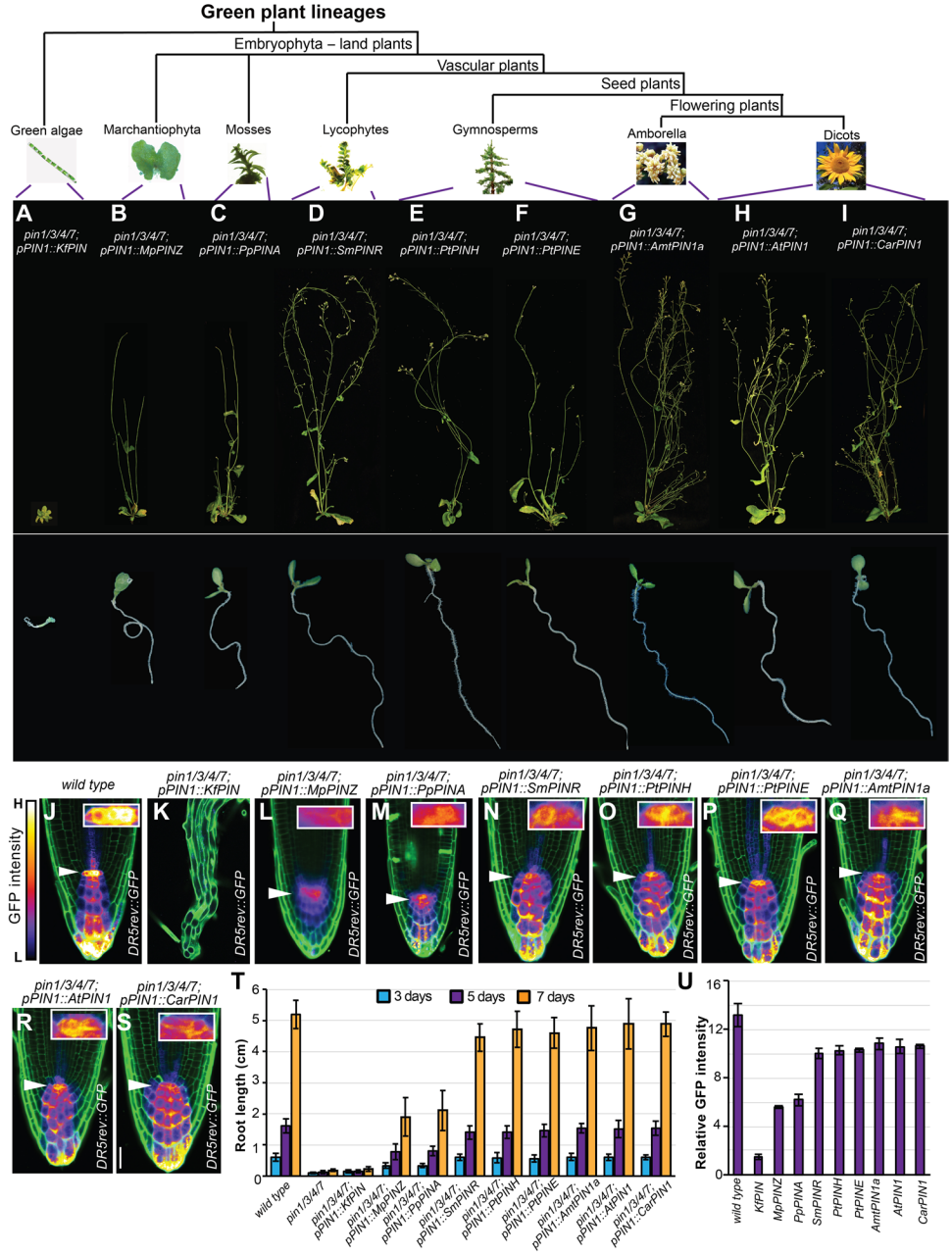

Fig. 4. PIN function in *Arabidopsis* shoot/root development originated at land plants. (**A** to **I**) The interspecies complementation experiments with homologous *PIN* genes from a green alga (*KfPIN*) (A), a marchantiophyte (*MpPINZ*) (B), a moss (*PpPINA*) (C), a lycophyte (*SmPINR*) (D), a gymnosperm (*PtPINH* and *PtPINE*) (E and F), and three flowering plants *Amborella trichopoda* (AmtPIN1a) (G), *Arabidopsis* (*AtPIN1*) (H), and *C. rubella* (*CarPIN1*) (I). Except for the green alga gene encoding KfPIN (A), all of the land plant canonical PIN genes were able to rescue the severe defects of *pin1/3/4/7* mutant in shoot/root development (B to I). Phenotypic analysis of the shoots with 7-week-old plants and the roots with 1-week-old seedlings. (**J** to **S**) Auxin maximum in root tips of the 7-day-old *Arabidopsis* PIN transformants in (A) to (I), indicated by synthetic auxin-responsive reporter *DR5rev::GFP*. The level of *DR5rev::GFP* expression is reflected by signal intensity. The GFP channel images are shown in pseudocolor, and the intensity scale is shown at the left (H, high; L, low). The white arrowhead indicates the quiescent center. Scale bar, 20 μm. (**T**) Quantification of root lengths of wild type, *pin1/3/4/7* mutant, and PIN transformants in (A) to (I) after growing for 3, 5, and 7 days, respectively. Data represent means ± SD ($n = 11$ roots from each transgenic line). (**U**) Quantification of the *DR5rev::GFP* signal intensity in wild type, *pin1/3/4/7* mutant, and transgenic lines in (J) to (S). Data represent means ± SD ($n = 6$ roots from each transgenic line).

were unable to rescue the *pin1* defect in flower development (Fig. 5, D to F). These transgenic lines only produced aberrant flowers with almost no stamens and an excessive number of petals. Therefore, they were infertile and failed to produce viable seeds in the siliques (Fig. 5, J to L, and fig. S9A). The plausible *PIN1* gene (*AmtPIN1a*) identified from *A. trichopoda*, a most primitive living flowering plant (*4*), could largely rescue the defective flower in *Arabidopsis pin1* mutant, producing functional flowers with stamens and viable seeds in the siliques, although the seed number was much lower than that in the wild type (Fig. 5L and fig. S9A). The *PIN1* genes from other flowering plants

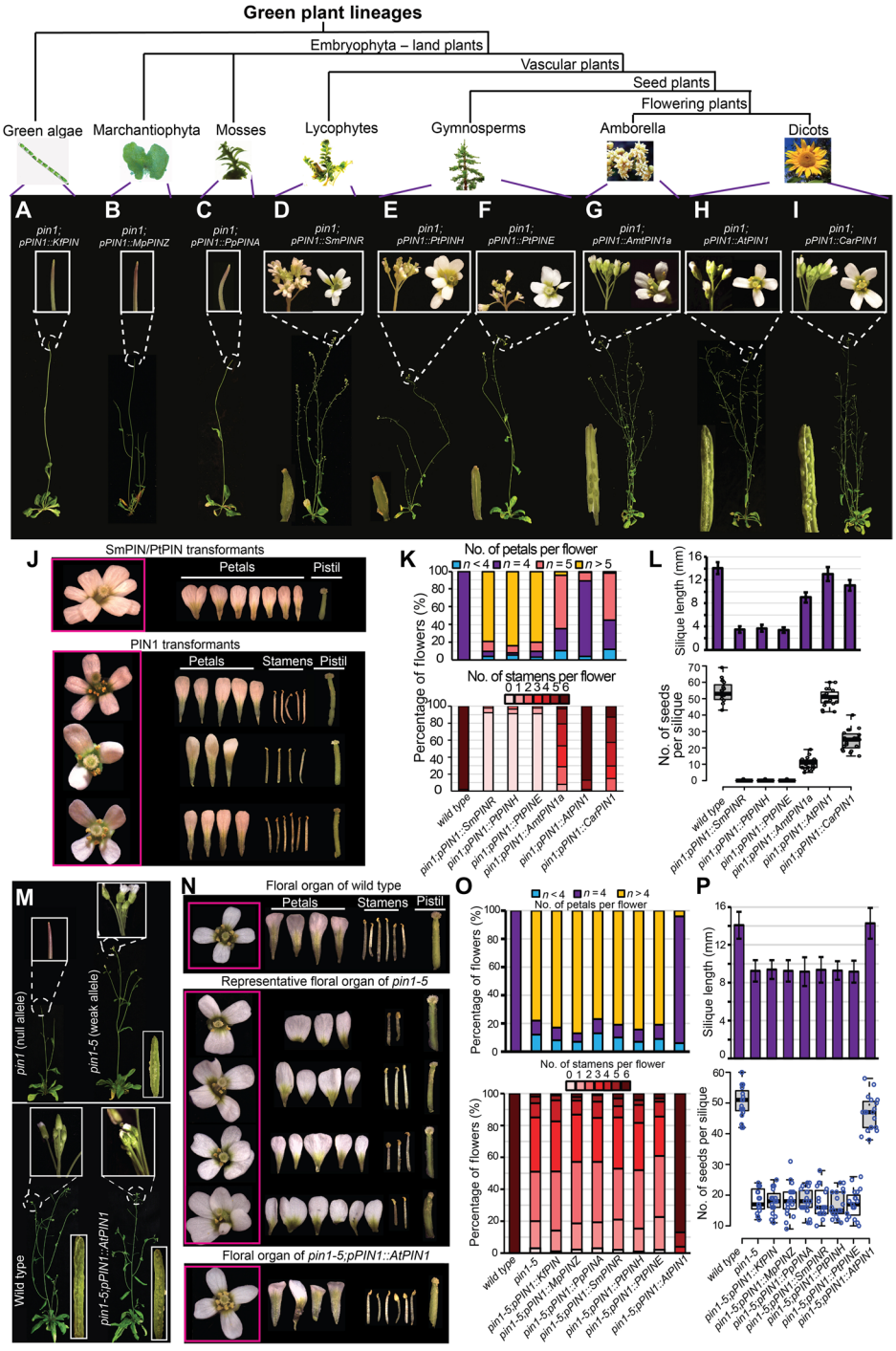

Fig. 5. PIN function in *Arabidopsis* floral organ development emerged at flowering plants. (**A** to **I**) The interspecies complementation experiments with homologous *PIN* genes from a green alga (*KfPIN*) (A), a marchantiophyte (*MpPINZ*) (B), a moss (*PpPINA*) (C), a lycophyte (*SmPINR*) (D), a gymnosperm (*PtPINH* and *PtPINE*) (E and F), and three flowering plants *A. trichopoda* (*AmtPIN1*) (G), *Arabidopsis* (*AtPIN1*) (H), and *C. rubella* (*CarPIN1*) (I). Only the flowering plant genes encoding *AmtPIN1a*, *AtPIN1*, and *CarPIN1* from the PIN1 clade were able to rescue the *Arabidopsis pin1* defects in floral organ formation and thus led to seed formation in the siliques (G to I). The canonical PINs from vascular plants (lycophyte *SmPINR*, gymnosperm *PtPINE*, and *PtPINH*) and flowering plants (*AmtPIN1*, *AtPIN1*, and *CarPIN1*) have the capacity to rescue the defective phenotype of *Arabidopsis pin1* with naked inflorescence (D to I). Phenotypic analysis of the shoot system with 7-week-old plants. (**J**) Anatomical structure of the floral organ from PIN transformants in (A) to (I). $n = 100$ flowers from each transgenic line. (**K**) Statistical analysis of the flower pattern of PIN transformants in (A) to (I). (**L**) Silique length and seed number per silique concerning these PIN transformants in (A) to (I). $n = 13$ to 19 siliques from each transgenic line. (**M**) Phenotype of the inflorescence in weak allele *pin1-5* mutant, null allele *pin1* mutant, wild type, and *pin1-5;pPIN1::AtPIN1* transgenic line. (**N**) Anatomical structure of the floral organ from wild type, *pin1-5*, and *pin1-5;pPIN1::AtPIN1* transgenic line. (**O**) Statistical analysis of the flower pattern of the PIN transformants in *pin1-5* mutant background. $n = 100$ flowers from each transgenic line. (**P**) Silique length and seed number per silique concerning these PIN transformants in the *pin1-5* mutant background. $n = 13$ to 19 siliques from each transgenic line. Photo credit: Yuzhou Zhang, Institute of Science and Technology (IST) Austria.

A. thaliana (AtPIN1) and *C. rubella* (*CarPIN1*) successfully rescued the *Arabidopsis pin1* mutant phenotype, and so fertile flowers and viable seeds in their siliques were produced (Fig. 5, H to L, and fig. S9).

Notably, the failed complementation of naked inflorescence of *Arabidopsis* null mutant *pin1* by green algal *KfPIN* and *PIN* genes (*MpPINZ* and *PpPINA*) from early diverging extant land plant precluded our further evaluation of their function in *Arabidopsis* floral organ development (Fig. 5, A to C). Therefore, we repeated the interspecies genetic complementation experiments using the weak *pin1-5* mutant allele (*37*), which displayed a normally shaped inflorescence but impaired flower development with a reduced number of stamens, frequently increased number of petals, and significantly decreased seed set (Fig. 5, M to P, and fig. S9B). The complementation experiment showed that all of the *PIN* genes (*KfPIN*, *MpPINZ*, and *PpPINA*) from basal plant lineages were still incapable of rescuing the *pin1*-5 defects in flower development (Fig. 5O), showing similar flower patterns to that of *pin1-5* mutant. Similarly, the *SmPINR* from the lycophyte and *PIN* genes (*PtPINE* and *PtPINH*) from the seed plant *P. taeda* were also unable to restore the *pin1-5* defects in flower development (Fig. 5O), whereas the *Arabidopsis PIN1* successfully restored the defective floral organ of *pin1-5* mutant and thus produced far more viable seeds in siliques than transgenic lines carrying nonflowering plant PINs (Fig. 5, N to P).

These results were consistent with our genetic complementation results with the null *pin1* mutant allele and implied that the functional PINs to regulate *Arabidopsis* floral organ formation might emerge in flowering plants after their divergence from the basal seed plant lineage.

PIN polar localization contributes to their divergent function in *Arabidopsis* patterning

The polar localization of PINs in cells plays a crucial role in plant patterning (*12*, *26*), which is achieved by determining the directionality of auxin flow for auxin gradient establishment. This prompted us to ask whether the functional differences of these PIN orthologs in regulating *Arabidopsis* tissue/organ formation are linked to the differences in their cellular polarities in *Arabidopsis* cells.

To reveal the polarity of these heterologous PIN members in *Arabidopsis*, we fused a *GFP* tag to these orthologous *PIN* genes and then expressed them under the control of *AtPIN1* promoter to analyze their subcellular localization in *Arabidopsis* root and inflorescence tissues (figs. S10 and S11). In root tissue, in contrast to the prominently basal cellular localization of AtPIN1 (Fig. 6G), the green algal KfPIN-GFP was evenly localized to the PMs of *Arabidopsis* root stele cells without polarity (Fig. 6A).

All the land plant GFP-fused canonical PIN proteins (MpPINZ, PpPINA, SmPINR, PtPINH, PtPING, and AtPIN1) showed basal PM localization in root stele cells (Fig. 6, B to G). However, compared with the vascular plant PIN proteins (SmPINR, PtPINH, PtPING, and AtPIN1), the nonvascular land plant PINs (MpPINZ and PpPINA) showed less polar localization in root stele cells (Fig. 6H). These findings were in accordance with the results of auxin maximum analysis by *DR5rev::GFP* in root tips and interspecies genetic complementation analysis of PIN function in *Arabidopsis* root development (Fig. 4). In conclusion, these results suggest that the polarity differences of these PIN orthologs in *Arabidopsis* root stele cells determine their differential function in regulating *Arabidopsis* root development.

In addition, we examined the cellular polarity of these PIN orthologs in the *Arabidopsis* inflorescence. All the GFP-fused canonical PIN proteins (SmPINR, PtPINH, and PtPING) from the vascular plants were polarly localized to the PM of the epidermal cells in *Arabidopsis* inflorescence shoot apical meristem as seen for AtPIN1-GFP (fig. S11, A to D). In contrast, the green algal KfPIN-GFP and nonvascular land plant PIN-GFP proteins (MpPINZ-GFP and PpPINA-GFP) showed hardly any polar localization in *Arabidopsis* inflorescence epidermis (fig. S11, E to G). These results were consistent with the functional analysis of orthologous PIN in *Arabidopsis* inflorescence development by interspecies genetic complementation experiments (Fig. 5, A to I) and imply that the polarity of these orthologous PINs defines their function in *Arabidopsis* inflorescence development.

Notably, as compared with *Arabidopsis* PIN1, these PINs from basal plants showed very weak or no polarity in *Arabidopsis* cells; nevertheless, some have been reported to be polarly localized in these basal plants, such as the moss PpPINA and possibly also the PIN from the green alga *Chara vulgaris* (*22*, *38*). These results imply that the functional partners from the basal plant lineages, which regulate their own polarity in basal plants, are missing or are functionally modified in the flowering plant *Arabidopsis* during their coevolution process with PIN proteins. This would explain the failure to establish polarity of the basal plant PINs, when they are heterologously expressed in *Arabidopsis* cells.

The evolutionary conservation of subcellular trafficking and phosphorylation of PM-localized PIN

In *Arabidopsis*, the subcellular localization of PIN auxin transporters to the PM (e.g., canonical AtPIN1) or to the ER (e.g., noncanonical AtPIN5) relies on the different subcellular trafficking pathways and PIN phosphorylation status (*14*, *39*, *40*). Because the orthologous PIN proteins from the green alga *Klebsormidium* (KfPIN) and basal land plants (e.g., PpPINA) were localized to PM in *Arabidopsis* cells, we proposed that these heterologous PINs undergo similar subcellular trafficking in *Arabidopsis* cells to AtPIN1, and not to AtPIN5.

To test this hypothesis, we treated the transgenic *Arabidopsis* roots harboring heterologous PINs from other plant species with brefeldin A (BFA; a vesicle-trafficking inhibitor). Different from the BFA-insensitive trafficking of the ER-localized PINs (*41*, *42*), we found that all these PM-localized orthologous PINs were able to aggregate and form intracellular BFA compartments in *Arabidopsis* cells (fig. S12, A to E), identical to the BFA-sensitive subcellular trafficking from endosomes to PM as known for the *Arabidop*sis PIN1. Furthermore, we applied BFA together with auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA), which interferes with the recycling of *Arabidopsis* PM-localized PIN proteins (e.g., AtPIN1), to preclude their BFA-induced aggregations (*43*, *44*). We found that TIBA also interfered with the BFA compartment formation of green algal KfPIN and other land plant PIN proteins in *Arabidopsis* root cells (fig. S12, F to J).

Moreover, to determine the phosphorylation states of basal plant PINs in *Arabidopsis*, total membrane proteins from the *Arabidopsis* transformants carrying PIN:GFPs (AtPIN1, AtPIN5, green algal KfPIN, and moss PpPINA) were isolated, and then we analyzed their phosphorylation using Phos-tag SDS–polyacrylamide gel electrophoresis (PAGE) (*45*). The phosphorylated protein shows a retarded migration and appears at a position corresponding to a higher molecular weight on the Phos-tag gel compared with its nonphosphorylated counterpart. Similar to the previous report (*41*), *Arabidopsis* ER-localized PIN5 failed to show any detectable band shift (fig. S13A).

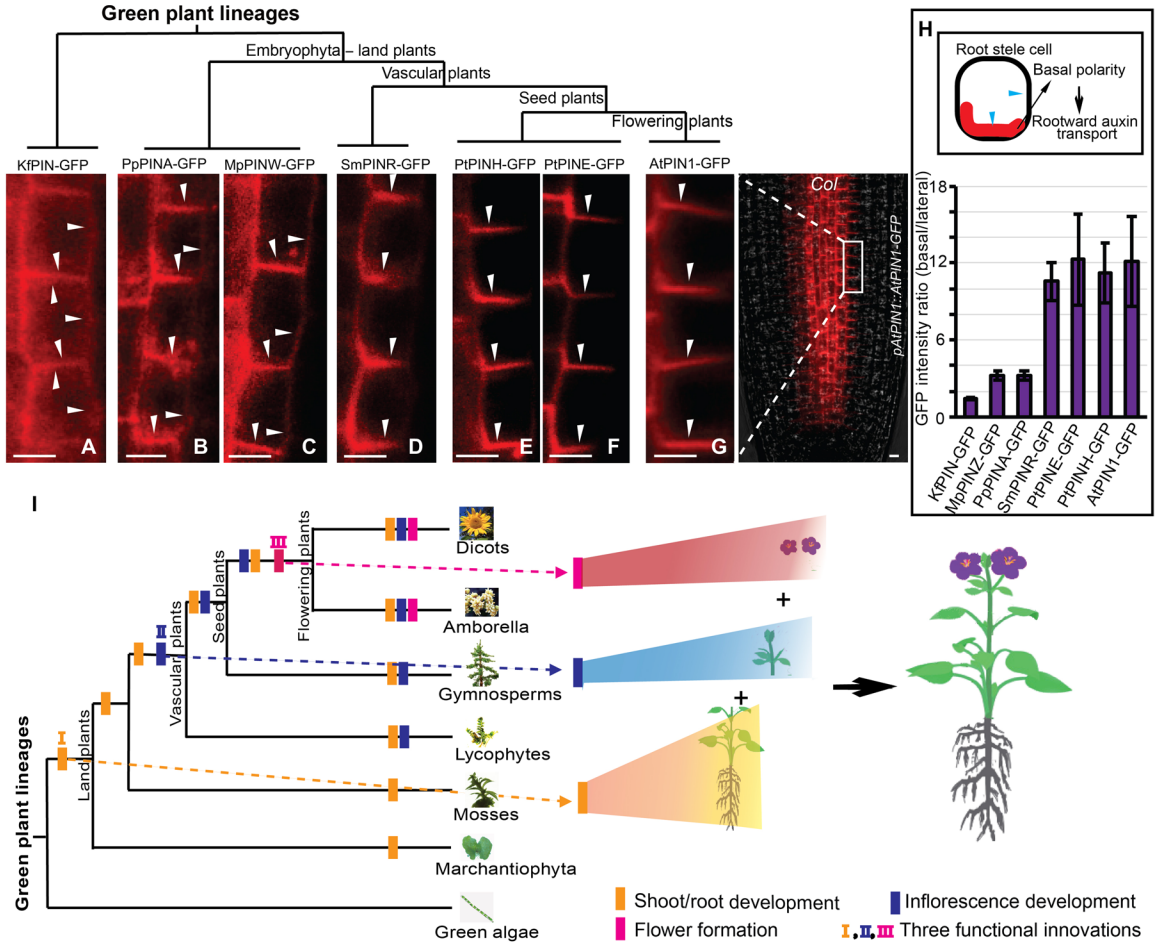

Fig. 6. The polarity of heterologous PIN in *Arabidopsis* root stele cells and the contribution of stepwise functional innovations of the PIN protein to the origin of flowering plants. (**A** to **G**) Cellular polarity analysis of KfPIN (A), MpPINZ (B), PpPINA (C), SmPINR (D), PtPINE (E), PtPINH (F), and AtPIN1 (G) in *Arabidopsis* root stele cells under the control of *Arabidopsis PIN1* promoter by fusion with GFP protein. The blue, yellow, and white arrowheads indicate the apical, basal, and lateral localization of PIN proteins in root stele cells, respectively. Scale bars, 10 μm. (**H**) Ratio of PIN-GFP intensity between the basal side and the designated lateral side toward the outside of the root. The blue arrowheads indicate the two cellular sides for analysis. Data represent means ± SD ($n = 10$ from each transgenic line). (**I**) Schematic showing the three-step functional innovation that occurred in the PIN protein during plant evolution. The three disparate functions of PIN (i.e., shoot/root development, inflorescence development, and floral organ formation), which evolved in three distinct plant evolution milestones (the origin of land plants, vascular plants, and flowering plants), are associated with the patterning and growth of flowering plants as exemplified by *Arabidopsis*, implying the indispensable contribution of PIN evolution to the origin of flowering plants.

However, the PM-localized KfPIN and PpPINA showed obvious band shifts as AtPIN1 did (fig. S13, B to D). To test whether the observed shift in molecular weight resulted from the phosphorylation, we incubated these protein extracts with λ-phosphatase. We observed that the higher–molecular weight bands of basal plant PINs were sensitive to the λ-phosphatase treatment (fig. S13, B to D), demonstrating that these additional PIN:GFP signals with reduced mobility are the result of phosphorylation. Together, the basal plant PIN proteins such as KfPIN and PpPINA can be recognized and phosphorylated in *Arabidopsis*.

In summary, our results revealed that the *Arabidopsis* components, which are involved in the subcellular trafficking and phosphorylation of *Arabidopsis* PM-localized PINs, were still able to recognize and regulate orthologous PIN proteins from the green alga *Klebsormidium* and basal land plants, resulting in their subcellular localization to the PM of *Arabidopsis* cells. Therefore, we postulate that the components, associated with subcellular trafficking and phosphorylation of *Arabidopsis* canonical PINs, are functionally conserved in the regulation of PM-localized PINs from other basal plant lineages.

DISCUSSION

During plant evolution, one of the most important events was the emergence of flowering plants (*3*). Our work showed how the family of PIN auxin transporters, which are the key components of the auxin distribution network mediating numerous aspects of plant development (*6*, *14*), has evolved gradually by a series of functional innovations. Those acquired innovations are associated with morphological patterning of three essential architectural organs of the flowering plant *Arabidopsis*: shoot/root, inflorescence, and flower.

Three functional innovations of PIN family during plant evolution

Our work highlights the three functional innovations of PIN auxin transporters that occurred at three distinct plant evolutionary stages

(Fig. 6I): (i) The first innovation emerged at the origin of land plants after their divergence from the green algal lineage (*46*), and this acquired capacity was integrated into the control of shoot/root development of the flowering plant *Arabidopsis*. This is in accordance with the origin of the *PINOID (PID)–like* genes, essential for the *Arabidopsis* organ development by controlling PIN-generated auxin gradients and maxima. These genes were identified in representatives of early diverging land plants such as mosses but not in green algae (*47*). This suggests that the *PID-like* and *PIN* genes probably coevolved during the transition of plants from water to land. (ii) The second innovation of PINs took place in vascular plants after their divergence from the bryophyte lineages, leading to the acquisition of the function that was capable of maintaining the *Arabidopsis* inflorescence development. (iii) The third innovation may have emerged at the origin of the flowering plants. The latest emerged PIN clades, PIN1 and sister of PIN1 (SoPIN1), are found only in flowering plants (*21*) and are associated with floral organ formation as exemplified by *Arabidopsis*. In the future, revealing PIN functions in the developmental patterning of other plant species, as well as the cross-species genetic complementation with more and more other homologous *PIN* genes, would further test our proposed hypothesis.

The PIN1/SoPIN1 function of floral organ development in diverse flowering plant species

The floral organs are specifically present in flowering plants. They promote efficient fertilization and enable rapid reproductive capacity. Thus, flowering plant populations can spread faster than those of their predecessors (*4*). Our results, together with previously published data, support the view that the emergence of PIN1/SoPIN1 is of crucial importance for the origin of flowering plants. For example, the flowering plant *Cardamine hirsuta pin1* mutants also produced a reduced number of aberrant flowers on the stem and no siliques (*48*), and the *Medicago truncatula pin1* (or *slm1*) mutant showed impaired flower formation (*49*). This demonstrates that the PIN1 clade is required for floral organ development in other flowering plant species besides *Arabidopsis*. In addition, when the flowering plant maize (monocot) was treated with the PIN auxin transporter inhibitor *N*-1-naphthylphthalamic acid, it resulted in barren inflorescence, similar to the *Arabidopsis pin1* mutant phenotype (*50*). Very recently, it was shown that knocking out SoPIN1 in the flowering plant *Brachypodium* led to barren inflorescence similar to the *Arabidopsis pin1* mutant phenotype. However, the loss of PIN1 in *Brachypodium* only altered the stem growth rather than inducing inflorescence defects (*51*). In agreement with this result, only the *Brachypodium* SoPIN1, but not its PIN1, was able to complement the null *Arabidopsis pin1* mutant defects in flower formation. That leads to the assumption that during the evolution of flowering plants, the SoPIN1 might alternatively be selected to replace the PIN1 function and to regulate the floral organ formation in some flowering plants. Together, these findings indicate that PIN1/SoPIN1 function in floral organ/inflorescence formation is evolutionarily conserved across the flowering plant lineages and also confirm the crucial role of PIN1/SoPIN1 at the origin of the flowering plants.

Besides the macro-evolution of the polar auxin transporter PIN that results in the vast functional divergence of disparate PIN clades, the PIN members from the PIN1 clade also seem to have experienced a micro-evolution during the evolution of flowering plants, which led to the slightly functional divergence of PIN1 in controlling floral organ formation. For instance, the *PIN1* genes from other flowering plants *Amborella* and *Capsella* (*AmtPIN1* and *CarPIN1*) could greatly, but not completely, recover the *Arabidopsis pin1* mutant. In contrast to floral organs with stable four petals and six stamens in the wild type and *pin1;pPIN1::AtPIN1*, the flowers of transgenic lines with the complementation by *AmtPIN1* and *CarPIN1* showed occasionally a reduced number of stamens and frequently five or more petals (Fig. 5, G and I). This suggests that the micro-evolution of *PIN1* might have contributed to the evolution of diverse floral structures and has allowed them to adapt to various means of pollination/fertilization and variable environments during their conquest of the earth.

The contribution of motifs in the central HL to PIN functions

The PIN proteins are composed of a tripartite domain structure: predicted TMD at the N and C termini and a central HL domain. Functional analysis of the chimeric PIN (V1 and V2) proteins suggests that the canonical PINs might have been subfunctionalized by modifications of their HLs (Fig. 3F). Previously, it has been reported that phosphorylation of the middle serine of three repetitive conserved TPRXS(N/S) motifs within the HL plays a crucial role in the apical localization of PIN and its function in root gravitropism (*33*). However, the SmPINR and SoPIN1, lacking the third TPRXS(N/S) motif (fig. S14), are still able to recover the *pin1* naked inflorescence (Fig. 5) (*51*). This indicates that this motif is likely not necessary for the canonical PIN function in the inflorescence development of flowering plants. Some of the motifs, evolutionarily conserved in the HL of canonical PINs used for our complementation analysis, are lost or altered in the HL of PIN1/SoPIN1 (fig. S14), which might contribute to their exclusive function in the floral organ development. With the increasing availability of PIN sequences retrieved from more plant species and with unraveling their functions by means of genetic or molecular biology methods, it will be possible to establish an exact correspondence between the HL motifs and different aspects of PIN functions.

The evolutionary changes in both coding and regulatory regions of PIN members

There has been an intense debate that whether the cis-regulatory changes or the mutations in coding regions of the genes played a key role in the morphological innovations of creatures during evolution (*52*, *53*).

Our results suggest that the evolutionary innovations occurred in both the (i) PIN coding regions (e.g., HL domain), which determines the biological properties of PIN molecules, and (ii) their cis-regulatory region (gene promoter), which defines their expression regions (e.g., certain cell types or tissues). PIN polarity that determines PIN function in plant tissue/organ formation relies on the biological features of PIN proteins and the cell types where they are expressed (Figs. 3 and 6, B to G). Therefore, we proposed that this extensive diversification of PIN molecular properties and their different expression patterns enables the *PIN* gene family to acquire a variety of developmental roles in flowering plants, and thus contributes to the establishment of their complex architecture.

MATERIALS AND METHODS

Search for PIN family members

The *PIN* coding sequences (CDSs) in the following plants were identified using the *A. thaliana* PIN1 protein sequence as a query in

BLAST searches against the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST) for the following species: *M. polymorpha*, *P. patens*, *S. moellendorffii*, *C. rubella*, *A. thaliana*, *Oryza sativa*, *Zea mays*, and *Brachypodium arboreum*. We obtained the full-length KfPIN CDS from transcriptome sequences of *K. flaccidum* (UTEX strain #321). The PIN sequences of *Picea abies* and *P. taeda* were identified from the Spruce Genome Project database (http://congenie.org/start). The *PIN* CDS of *A. trichopoda* was identified from the EnsemblPlants database (https://plants.ensembl.org/Amborella_trichopoda/Info/Annotation/).

Whole-mount in situ immunolocalization

Immunolocalization of PIN1 and PIN2 proteins in 3-day-old primary roots of the wild-type and *pin1;pPIN1::PIN2* transgenic line was carried out as described previously (*54*) using anti-PIN1 or anti-PIN2 diluted 1:1000 as the primary antibody and goat anti-rabbit antibody coupled to Cy3 (Sigma-Aldrich) diluted 1:600 as the secondary antibody. Signal was observed using the Zeiss Inverted Confocal Laser Scanning Microscope 710. Three biological repeats were performed with similar results.

Plant materials and transformation, vector construction, and complementation analysis

The *Arabidopsis* loss-of-function mutants *pin1*, *pin2*, *pin1/pin2*, *pin3/4/7*, and *pin1/3/4/7* were previously described (*20*, *23*), and the weak allele *pin1-5* mutant was also previously reported (*37*). The *pin2/3/4/7* quadruple mutant was constructed by crossing *pin2* as a male parent with *pin3/4/7* (female parent). To generate plasmids for genetic complementation analysis, 3.2-kb *Arabidopsis PIN1* promoter and *PIN* CDS from different plant species were separately cloned into the Gateway entry vectors pDONR4P1r and pDONR221 by BP reaction, and then they were fused and cloned into the Gateway destination vector pB7m24GW.3 by LR reaction. The primers used to generate these constructs are shown in table S3. Transgenic *Arabidopsis* plants were generated using the floral dip method and selected on solid, half-strength Murashige and Skoog (MS) medium containing Basta (15 mg/ml; glufosinate).

For complementation analysis of PIN transformants with heterologous expression of other plant species *PIN* in *Arabidopsis*, we collected more than 20 independent transgenic lines for each construct. Then, the RNA from the individual transgenic line was extracted and reversely transcribed to complementary DNA (cDNA) for quantitative real-time polymerase chain reaction (qRT-PCR) analysis with the housekeeping gene *UBQ5* used as the internal standard. We selected these transgenic lines with the expression level of *PIN1* homologs comparable to that of *PIN1* in wild type for phenotypic analysis to rule out the possibility that the nonrecovered phenotype resulted from the insufficient expression of the *PIN* genes in *pin1* or *pin1/3/4/7* mutants.

Confocal imaging

For confocal microscopic analyses on *Arabidopsis* roots, 7-day-old seedlings grown in half-strength MS medium were stained with propidium iodide (10 μg/ml) for 5 min, washed briefly in ddH_2O, and visualized at 600 to 640 nm for propidium iodide and 500 to 560 nm for GFP on an LSM 710 NLO confocal microscope with Duoscan. The *DR5rev::GFP* signal intensity of the S1 layer of columella cells indicated by red arrows was quantified by the measurement of the mean gray value with the Fiji software (https://imagej.net/Fiji).

To image the PIN localization in *Arabidopsis* inflorescence, the primary shoot apical meristem of 5-week-old adult plants was dissected with flower bud and big floral primordia removed and then it was immersed in water and imaged by 40× water objective.

In vivo phosphorylation assay of PIN-GFP proteins

To analyze the phosphorylation state of GFP-tagged PIN proteins in planta, 10-day-old seedlings were ground in liquid nitrogen and vortexed vigorously in extraction buffer 1 [50 mM tris-HCl (pH 7.5), 150 mM NaCl, Complete EDTA-free protease inhibitor cocktail (Roche), and PhosSTOP phosphatase inhibitor cocktail (Roche)] in a 1:10 (w/v) ratio. The resulting homogenate was centrifuged at 20,000*g* for 30 min at 4°C. The pellet was resuspended in extraction buffer 2 [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.5% CHAPS, 1% Triton X-100, Complete EDTA-free protease inhibitor cocktail (Roche), and PhosSTOP phosphatase inhibitor cocktail (Roche)] and centrifuged at 12,000*g* for 20 min at 4°C. The supernatant was used for immunoprecipitation assay with anti-GFP microbeads according to the manufacturer's instructions (μMACS Epitope Tag Protein Isolation Kit, MACS Miltenyi Biotec). Before the elution step, phosphatase treatment was performed on the column to the corresponding samples, where GFP-tagged PIN proteins, bound to super-paramagnetic micro-MACS beads, were treated with preheated (30°C) reaction buffer containing 1 μl (400 U) of Lambda Protein Phosphatase (New England Biolabs) for 15 min. The reaction was stopped by washing the column with extraction buffer 1, followed by elution with Laemmli buffer containing 1 mM $ZnCl_2$. Samples were separated by Phos-tag SDS-PAGE [7.5% SuperSep Phos-tag (50 μM), https://labchem-wako.fujifilm.com/us/category/00899.html], transferred to a polyvinylidene difluoride membrane, and analyzed by immunoblot using primary anti-GFP, N-terminal antibody (Sigma-Aldrich) and secondary anti-rabbit horseradish peroxidase–conjugated antibody (1:5000, GE Healthcare). Detection was performed using the SuperSignal West Femto Maximum Sensitivity Substrate Detection Kit (Thermo Fisher Scientific). Images were taken using the image analyzer Amersham 600RGB (GE Healthcare), and quantification of the protein signal was performed using Fiji software.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/50/eabc8895/DC1

View/request a protocol for this paper from *Bio-protocol*.

REFERENCES AND NOTES

1. S. A. Rensing, Great moments in evolution: The conquest of land by plants. *Curr. Opin. Plant Biol.* **42**, 49–54 (2018).
2. C. F. Delwiche, E. D. Cooper, The evolutionary origin of a terrestrial flora. *Curr. Biol.* **25**, R899–R910 (2015).
3. E. Pennisi, On the origin of flowering plants. *Science* **324**, 28–31 (2009).
4. Amborella Genome Project, The *Amborella* genome and the evolution of flowering plants. *Science* **342**, 1241089 (2013).
5. I. Blilou, J. Xu, M. Wildwater, V. Willemsen, I. Paponov, J. Friml, R. Heidstra, M. Aida, K. Palme, B. Scheres, The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature* **433**, 39–44 (2005).
6. E. Benková, M. Michniewicz, M. Sauer, T. Teichmann, D. Seifertová, G. Jürgens, J. Friml, Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* **115**, 591–602 (2003).
7. S. Vanneste, J. Friml, Auxin: A trigger for change in plant development. *Cell* **136**, 1005–1016 (2009).
8. S. K. Mutte, H. Kato, C. Rothfels, M. Melkonian, G. K.-S. Wong, D. Weijers, Origin and evolution of the nuclear auxin response system. *eLife* **7**, e33399 (2018).

9. L. Gälweiler, C. Guan, A. Müller, E. Wisman, K. Mendgen, A. Yephremov, K. Palme, Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. *Science* **282**, 2226–2230 (1998).
10. J. Friml, E. Benková, I. Blilou, J. Wisniewska, T. Hamann, K. Ljung, S. Woody, G. Sandberg, B. Scheres, G. Jürgens, K. Palme, AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis*. *Cell* **108**, 661–673 (2002).
11. J. Petrášek, J. Mravec, R. Bouchard, J. J. Blakeslee, M. Abas, D. Seifertová, J. Wiśniewska, Z. Tadele, M. Kubeš, M. Čovanová, P. Dhonukshe, P. Skůpa, E. Benková, L. Perry, P. Křeček, O. R. Lee, G. R. Fink, M. Geisler, A. S. Murphy, C. Luschnig, E. Zažímalová, J. Friml, PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* **312**, 914–918 (2006).
12. J. Wisniewska, J. Xu, D. Seifartová, P. B. Brewer, K. Růžička, L. Blilou, D. Rouquié, E. Benková, B. Scheres, J. Friml, Polar PIN localization directs auxin flow in plants. *Science* **312**, 883 (2006).
13. J. J. Blakeslee, W. A. Peer, A. S. Murphy, Auxin transport. *Curr. Opin. Plant Biol.* **8**, 494–500 (2005).
14. M. Adamowski, J. Friml, PIN-dependent auxin transport: Action, regulation, and evolution. *Plant Cell* **27**, 20–32 (2015).
15. M. Pernisova, T. Prat, P. Grones, D. Harustiakova, M. Matonohova, L. Spichal, T. Nodzynski, J. Friml, J. Hejatko, Cytokinins influence root gravitropism via differential regulation of auxin transporter expression and localization in *Arabidopsis*. *New Phytol.* **212**, 497–509 (2016).
16. H. Rakusová, M. Abbas, H. Han, S. Song, H. S. Robert, J. Friml, Termination of shoot gravitropic responses by auxin feedback on PIN3 polarity. *Curr. Biol.* **26**, 3026–3032 (2016).
17. K. Okada, J. Ueda, M. K. Komaki, C. J. Bell, Y. Shimura, Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. *Plant Cell* **3**, 677–684 (1991).
18. H. S. Robert, C. Park, C. L. Gutièrrez, B. Wójcikowska, A. Pěnčík, O. Novák, J. Chen, W. Grunewald, T. Dresselhaus, J. Friml, T. Laux, Maternal auxin supply contributes to early embryo patterning in *Arabidopsis*. *Nat. Plants* **4**, 548–553 (2018).
19. P. Žádníková, K. Wabnik, A. Abuzeineh, M. Gallemi, D. Van Der Straeten, R. S. Smith, D. Inzé, J. Friml, P. Prusinkiewicz, E. Benková, A model of differential growth-guided apical hook formation in plants. *Plant Cell* **28**, 2464–2477 (2016).
20. J. Le, X.-G. Liu, K.-Z. Yang, X.-L. Chen, J.-J. Zou, H.-Z. Wang, M. Wang, S. Vanneste, M. Morita, M. Tasaka, Z.-J. Ding, J. Friml, T. Beeckman, F. Sack, Auxin transport and activity regulate stomatal patterning and development. *Nat. Commun.* **5**, 3090 (2014).
21. T. Bennett, S. F. Brockington, C. Rothfels, S. W. Graham, D. Stevenson, T. Kutchan, M. Rolf, P. Thomas, G. K.-S. Wong, O. Leyser, B. J. Glover, C. J. Harrison, Paralogous radiations of PIN proteins with multiple origins of noncanonical PIN structure. *Mol. Biol. Evol.* **31**, 2042–2060 (2014).
22. T. Viaene, K. Landberg, M. Thelander, E. Medvecka, E. Pederson, E. Feraru, E. D. Cooper, M. Karimi, C. F. Delwiche, K. Ljung, M. Geisler, E. Sundberg, J. Friml, Directional auxin transport mechanisms in early diverging land plants. *Curr. Biol.* **24**, 2786–2791 (2014).
23. C. Luschnig, R. A. Gaxiola, P. Grisafi, G. R. Fink, EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. *Genes Dev.* **12**, 2175–2187 (1998).
24. T. Bennett, G. Hines, M. van Rongen, T. Waldie, M. G. Sawchuk, E. Scarpella, K. Ljung, O. Leyser, Connective auxin transport in the shoot facilitates communication between shoot apices. *PLOS Biol.* **14**, e1002446 (2016).
25. J. Kleine-Vehn, Z. Ding, A. R. Jones, M. Tasaka, M. T. Morita, J. Friml, Gravity-induced PIN transcytosis for polarization of auxin fluxes in gravity-sensing root cells. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 22344–22349 (2010).
26. J. Friml, A. Vieten, M. Sauer, D. Weijers, H. Schwarz, T. Hamann, R. Offringa, G. Jürgens, Efflux-dependent auxin gradients establish the apical–basal axis of *Arabidopsis*. *Nature* **426**, 147–153 (2003).
27. J. Felten, A. Kohler, E. Morin, R. P. Bhalerao, K. Palme, F. Martin, F. A. Ditengou, V. Legué, The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and arabidopsis through auxin transport and signaling. *Plant Physiol.* **151**, 1991–2005 (2009).
28. S. Sabatini, D. Beis, H. Wolkenfelt, J. Murfett, T. Guilfoyle, J. Malamy, P. Benfey, O. Leyser, N. Bechtold, P. Weisbeek, B. Scheres, An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. *Cell* **99**, 463–472 (1999).
29. J. Friml, J. Wiśniewska, E. Benková, K. Mendgen, K. Palme, Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. *Nature* **415**, 806–809 (2002).
30. Y. Zhang, G. Xiao, X. Wang, X. Zhang, J. Friml, Evolution of fast root gravitropism in seed plants. *Nat. Commun.* **10**, 3480 (2019).
31. P. Křeček, P. Skůpa, J. Libus, S. Naramoto, R. Tejos, J. Friml, E. Zažímalová, The PIN-FORMED (PIN) protein family of auxin transporters. *Genome Biol.* **10**, 249 (2009).
32. I. C. R. Barbosa, U. Z. Hammes, C. Schwechheimer, Activation and polarity control of PIN-FORMED auxin transporters by phosphorylation. *Trends Plant Sci.* **23**, 523–538 (2018).
33. P. Dhonukshe, F. Huang, C. S. Galvan-Ampudia, A. P. Mähönen, J. Kleine-Vehn, J. Xu, A. Quint, K. Prasad, J. Friml, B. Scheres, R. Offringa, Plasma membrane-bound AGC3 kinases phosphorylate PIN auxin carriers at TPRXS(N/S) motifs to direct apical PIN recycling. *Development* **137**, 3245–3255 (2010).
34. J. Zhang, T. Nodzyński, A. Pěnčík, J. Rolčík, J. Friml, PIN phosphorylation is sufficient to mediate PIN polarity and direct auxin transport. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 918–922 (2010).
35. P. Grones, M. Abas, J. Hajný, A. Jones, S. Waidmann, J. Kleine-Vehn, J. Friml, PID/WAG-mediated phosphorylation of the *Arabidopsis* PIN3 auxin transporter mediates polarity switches during gravitropism. *Sci. Rep.* **8**, 10279 (2018).
36. R. Skokan, E. Medvecká, T. Viaene, S. Vosolsobě, M. Zwiewka, K. Müller, P. Skůpa, M. Karady, Y. Zhang, D. P. Janacek, U. Z. Hammes, K. Ljung, T. Nodzyński, J. Petrášek, J. Friml, PIN-driven auxin transport emerged early in streptophyte evolution. *Nat. Plants.* **5**, 1114–1119 (2019).
37. S. Bencivenga, S. Simonini, E. Benková, L. Colombo, The transcription factors BEL1 and SPL are required for cytokinin and auxin signaling during ovule development in *Arabidopsis*. *Plant Cell* **24**, 2886–2897 (2012).
38. A. Żabka, J. T. Polit, K. Winnicki, P. Paciorek, J. Juszczak, M. Nowak, J. Maszewski, PIN2-like proteins may contribute to the regulation of morphogenetic processes during spermatogenesis in *Chara vulgaris*. *Plant Cell Rep.* **35**, 1655–1669 (2016).
39. M. Zwiewka, V. Bilanovičová, Y. W. Seifu, T. Nodzyński, The nuts and bolts of PIN auxin efflux carriers. *Front. Plant Sci.* **10**, 985 (2019).
40. C. Luschnig, G. Vert, The dynamics of plant plasma membrane proteins: PINs and beyond. *Development* **141**, 2924–2938 (2014).
41. A. Ganguly, M. Park, M. S. Kesawat, H.-T. Cho, Functional analysis of the hydrophilic loop in intracellular trafficking of *Arabidopsis* PIN-FORMED proteins. *Plant Cell* **26**, 1570–1585 (2014).
42. J. Mravec, P. Skůpa, A. Bailly, K. Hoyerová, P. Křeček, A. Bielach, J. Petrášek, J. Zhang, V. Gaykova, Y.-D. Stierhof, P. I. Dobrev, K. Schwarzerová, J. Rolčík, D. Seifertová, C. Luschnig, E. Benková, E. Zažímalová, M. Geisler, J. Friml, Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. *Nature* **459**, 1136–1140 (2009).
43. P. Dhonukshe, I. Grigoriev, R. Fischer, M. Tominaga, D. G. Robinson, J. Hašek, T. Paciorek, J. Petrášek, D. Seifertová, R. Tejos, L. A. Meisel, E. Zažímalová, T. W. J. Gadella, Y.-D. Stierhof, T. Ueda, K. Oiwa, A. Akhmanova, R. Brock, A. Spang, J. Friml, Auxin transport inhibitors impair vesicle motility and actin cytoskeleton dynamics in diverse eukaryotes. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 4489–4494 (2008).
44. N. Geldner, J. Friml, Y.-D. Stierhof, G. Jürgens, K. Palme, Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**, 425–428 (2001).
45. E. Kinoshita, E. Kinoshita-Kikuta, T. Koike, Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. *Nat. Protoc.* **4**, 1513–1521 (2009).
46. T. Viaene, C. F. Delwiche, S. A. Rensing, J. Friml, Origin and evolution of PIN auxin transporters in the green lineage. *Trends Plant Sci.* **18**, 5–10 (2013).
47. C. S. Galván-Ampudia, R. Offringa, Plant evolution: AGC kinases tell the auxin tale. *Trends Plant Sci.* **12**, 541–547 (2007).
48. M. Barkoulas, A. Hay, E. Kougioumoutzi, M. Tsiantis, A developmental framework for dissected leaf formation in the *Arabidopsis* relative *Cardamine hirsuta*. *Nat. Genet.* **40**, 1136–1141 (2008).
49. C. Zhou, L. Han, C. Hou, A. Metelli, L. Qi, M. Tadege, K. S. Mysore, Z.-Y. Wang, Developmental analysis of a *Medicago truncatula smooth leaf margin1* mutant reveals context-dependent effects on compound leaf development. *Plant Cell* **23**, 2106–2124 (2011).
50. X. Wu, P. McSteen, The role of auxin transport during inflorescence development in maize (*Zea mays*, Poaceae). *Am. J. Bot.* **94**, 1745–1755 (2007).
51. D. L. O'Connor, S. Elton, F. Ticchiarelli, M. M. Hsia, J. P. Vogel, T. Leyser, Cross-species functional diversity within the PIN auxin efflux protein family. *eLife* **6**, e31804 (2017).
52. E. Pennisi, Deciphering the genetics of evolution. *Science* **321**, 760–763 (2008).
53. P. J. Wittkopp, G. Kalay, *Cis*-regulatory elements: Molecular mechanisms and evolutionary processes underlying divergence. *Nat. Rev. Genet.* **13**, 59–69 (2012).
54. M. Sauer, J. Friml, Immunolocalization of proteins in plants. *Methods Mol. Biol.* **655**, 253–263 (2010).

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