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PINOID-centered genetic interactions mediate auxin action in cotyledon formation

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Abstract

Auxin plays a key role in plant growth and development through auxin local synthesis, polar transport, and auxin signaling. Many previous reports on *Arabidopsis* have found that various types of auxin-related genes are involved in the development of the cotyledon, including the number, symmetry, and morphology of the cotyledon. However, the molecular mechanism by which auxin is involved in cotyledon formation remains to be elucidated. *PID*, which encodes a serine/threonine kinase localized to the plasma membrane, has been found to phosphorylate the PIN1 protein and regulate its polar distribution in the cell. The loss of function of *pid* resulted in an abnormal number of cotyledons and defects in inflorescence. It was interesting that the *pid* mutant interacted synergistically with various types of mutant to generate the severe developmental defect without cotyledon. *PID* and these genes were indicated to be strongly correlated with cotyledon formation. In this review, *PID*-centered genetic interactions, related gene functions, and corresponding possible pathways are discussed, providing a perspective that PID and its co-regulators control cotyledon formation through multiple pathways.

KEYWORDS
auxin, cotyledon formation, PID, synergistic interaction

1 | INTRODUCTION

In *Arabidopsis*, the initiation of cotyledons at the transition stage of embryogenesis requires many critical genes with spatially and temporally differential expression, hormones at appropriate concentrations, and key proteins in the corresponding functional state to guarantee accurate cell division and proper pattern formation within the apical region of the embryo. The mechanism governing the timing and circumstances that lead to the initiation and formation of cotyledons is still unclear. However, over the past two decades, the discovery of the function of many important genes has given us a preliminary understanding of the development of cotyledons in *Arabidopsis*. The vast majority of these discovered genes are associated with auxin.

Auxin, as a small molecule, is indispensable for almost every aspect of plant growth and development. The formation and development of cotyledons in *Arabidopsis* are greatly affected by variations in auxin concentration, phosphorylation status and subcellular location of auxin carriers, and the activity of proteins involved in auxin signaling inside cells (Cheng et al., 2008; Cheng, Dai, & Zhao, 2007; Friml et al., 2004). The effects of several auxin-related genes such as *YUC* (*YUCCA*), *PIN* (*PIN-FORMED*), *PID* (*PINOID*), and *ARF* (*Auxin Response Factor*) on cotyledon formation and development are

persuasive since mutations in these genes generate aberrant cotyledon phenotypes. The *pid* mutant resulted in no cotyledon, an extremely severe developmental defect in *Arabidopsis* when combined with various mutations in *PIN1*, *NPY1*, *MOB1A*, *VPS28A*, *MAB1*, *MAB2*, *SAC7*, *PID* homologs, *ERFs*, *YUC*, or *TAA*, respectively (Cheng et al., 2008; Cheng, Qin, et al., 2007; Cui et al., 2016; Dhonukshe et al., 2010; Furutani et al., 2004; Ito et al., 2011; Liu et al., 2020; Ohbayashi et al., 2019; Song et al., 2021; Won et al., 2011). This highlights the crucial role *PID* plays in the formation of cotyledons. It is necessary to investigate the role of PID and its co-regulators in the development of cotyledons. This review will discuss genetic networks centered on *PID* in cotyledon formation to dissect the underlying mechanism.

2 | PID AND ITS HOMOLOGS FUNCTION IN COTYLEDON DEVELOPMENT

PID, a serine/threonine kinase located on the plasma membrane, has been shown to be involved in auxin polar transport, auxin signaling, and photomorphogenesis (Friml et al., 2004; Lin et al., 2017; Saini et al., 2017; Xu et al., 2019). *PID* is important for the development of cotyledons and inflorescences, as evidenced by the severe developmental defect phenotypes displayed in a variety of *pid* mutants in *Arabidopsis*, such as a high percentage of seedlings with three cotyledons and a small percentage with one cotyledon, as well as pin-like inflorescences in the mature stage. Strong *pid* mutants, such as *pid-1* and *pid-3*, produce a small but statistically significant percentage of seedlings with no cotyledons, while weak mutants *pid-2* and *pid-101* do not. The *pid-7.1.2.6* mutant displayed up to 11.4% of the no cotyledon phenotype. By comparing the location of the mutations and cotyledon defect phenotypes among *pid-1*, *pid-2*, *pid-3*, *pid-101*, and *pid-7.1.2.6*, the effect of these *pid* mutants on cotyledon formation appears to have little relationship with the location of the mutation sites in *PID* except *pid-7.1.2.6* (Figure 1). The truncated protein encoded by *pid-7.1.2.6* may be specific and play a negative regulatory role in the formation of cotyledons. Another possibility is that one or more mutations in other unknown genes in the *pid-7.1.2.6* line

genetically interact with *pid* to produce no cotyledon phenotype with a ratio of 11.4%. This requires a complementary experiment to confirm.

In *Arabidopsis*, *PID* has three closest homologs, which are *PID2*, *WAG1*, and *WAG2*. Their transcripts have been detected at stages of embryonic development (Cheng et al., 2008; Christensen et al., 2000; Furutani et al., 2004). The proteins encoded by these four genes contained a protein kinase domain, which made up the majority of the protein (Figure 2). There are no apparent developmental abnormalities caused by the *pid2*, *wag1*, or *wag2* T-DNA insertion mutants during the embryonic and flowering phases, in contrast to the *pid-101* mutant, which is a significant distinction between *pid* and mutations in its homologs. Both *pid-101 wag1* and *pid-101 wag2*, except *pid-101 pid2* double mutants, caused a small percentage of no cotyledon phenotype, whereas *pid-101 wag1 wag2* triple mutants dramatically increased the percentage of the no cotyledon phenotype absent from *pid2 wag1 wag2* (Bennett et al., 1995; Cheng et al., 2008; Dhonukshe et al., 2010). As a result, *PID* played a main role and overlapped with *WAG1* and *WAG2* in the formation of cotyledons. *PID2* appeared to have no relationship with the development of cotyledons. There are two explanations for this. Firstly, its function in cotyledon development has been completely substituted by *PID*, *WAG1*, and *WAG2*. Secondly, the T-DNA (SAIL_269_G07) insertion is located in the last exon of *PID2*. The majority of the protein kinase domain was still retained and could be partially functional in *pid2* (Figure 2). It may be part of the cause of the normal morphological character of the cotyledon in *pid2 wag1 wag2*.

PID, in conjunction with WAG1 or WAG2, has been found to phosphorylate PIN proteins to regulate their polar position, which is essential for the establishment of auxin concentration gradients and facilitates the initiation of tissue development. Adjustable localization of PIN proteins occurs in response to environmental stimuli or signals (Dhonukshe et al., 2010; Ding et al., 2011; Friml et al., 2002; Harrison & Masson, 2008; Rakusová et al., 2011). The PID activity can be inhibited by the second messenger Ca^{2+} or enhanced by a phospholipid signaling-related protein called PDK1 (3-phosphoinositide-dependent protein kinase 1) (Anthony et al., 2004; Zegzouti et al., 2006). It tended to propose a model in which
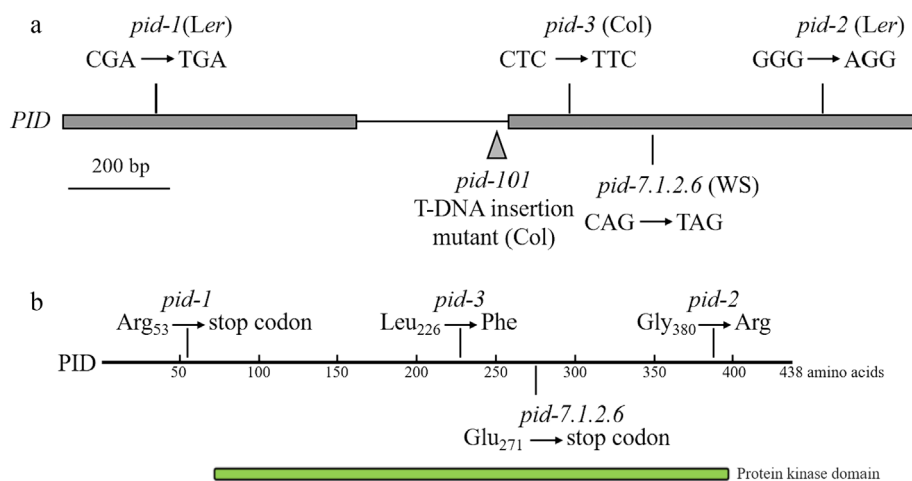

FIGURE 1 The structure of *PID* gene and domain in PID protein. (a) The location of different *pid* mutants. (b) The *pid* mutants lead to different amino acid changes.
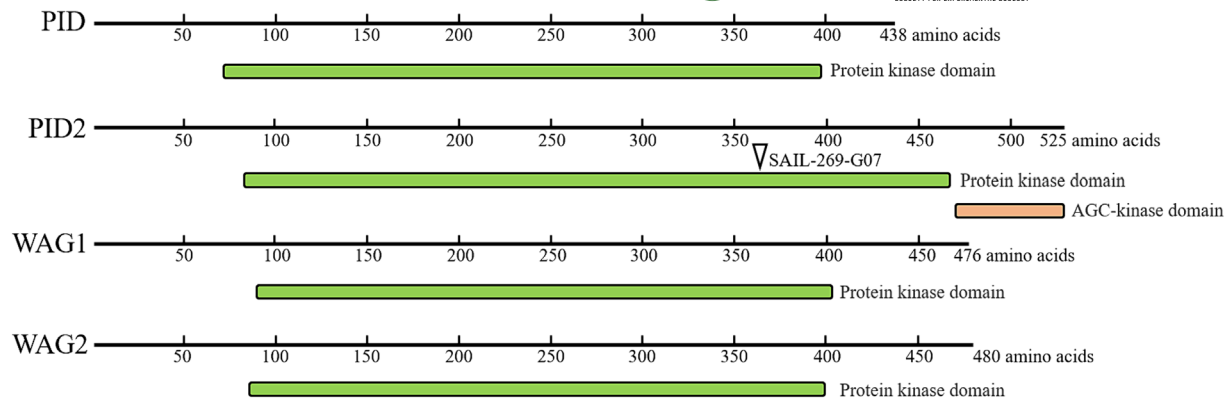
FIGURE 2 The domains in PID protein and its homologs.

PID-WAG1-WAG2 phosphorylate their substrates, such as PIN proteins, to alter the localization of proteins or activate one or more pathways in cells, to initiate cotyledon formation when their upstream receptors receive some kind of signal and then change their kinase activity directly or indirectly in cells on both sides of the apical embryo at the transition stage.

3 | PID AND ITS CO-REGULATORS FUNCTION IN COTYLEDON FORMATION

Among these genes that genetically interact with the *PID* associated with cotyledon formation, they belong to three main types according to their function in the auxin field: auxin biosynthesis, auxin polar transport, and auxin response.

3.1 | Auxin biosynthesis-related genes

There are currently two pathways identified in plants for the production of indole-3-acetic acid (IAA): Trp-dependent and Trp-independent pathways (Brumos et al., 2014; Kasahara, 2016). The former contains multiple branches, of which the TAA (TRYPTOPHAN AMINOTRANS-FERASE OF ARABIDOPSIS) and the YUC-catalyzed pathway have been clearly elucidated as the main form of IAA synthesis (Kasahara, 2016; Zhao, 2018). The pathway consists of two sequential chemical reactions: The front reaction converts Trp to IPA (indole-3-pyruvate), catalyzed by aminotransferases encoded by the *TAA* family, and flavin monooxygenases encoded by YUCs oxidize IPA to generate IAA in the subsequent reaction (Cheng, Dai, & Zhao, 2007; Won et al., 2011).

3.1.1 | YUC

In *Arabidopsis*, the *YUC* family has 11 members named *YUC1-YUC11* (Cheng et al., 2006). The 11 YUC proteins were briefly classified into two types based on differences in their subcellular localization of the protein and their tissue-specific distribution. One type is a kind of shoot-specific expressed and cytosol-localized proteins, including

YUC1, YUC2, YUC4, YUC6, YUC10, and YUC11; the other type, which contains YUC3, YUC5, YUC7, YUC8, and YUC9, is localized to the endoplasmic reticulum (ER) membrane and expressed in the root (Blakeslee et al., 2019; Kriechbaumer et al., 2016). While none of the single *yuc* mutants showed obvious developmental abnormalities, the combinations of several *YUC* gene mutations showed a variety of phenotypic disorders. For example, homozygous *yuc1 yuc2 yuc4 yuc6* quadruple mutants in the adult stage showed significant deficiencies in the acrial parts, especially the flower organs, and homozygous *yuc3 yuc5 yuc7 yuc8 yuc9* quintuple showed abnormity in root length and gravitropism, while *yuc1 yuc4 yuc10 yuc11* quadruple mutants had a rootless and without hypocotyl phenotype similar to that of *arf5* and *pin1 3 4 7* quadruple mutants, and most of them had only one cotyledon (Chen et al., 2014; Cheng et al., 2006; Cheng, Dai, & Zhao, 2007). It was clear that *YUCs* play a redundant role in plant growth and development, particularly in embryogenesis, as their expression pattern is highly correlated with the development of the corresponding tissue. Although local auxin biosynthesis, transport, and signaling were carried out by different gene families, some mutants among these genes showed similar defects during embryogenesis. For example, *yuc1 yuc4 yuc10 yuc11*, *pin1*, *pid*, and *arf5* had a single-cotyledon phenotype. There were some synergistic interactions among the combinations of different types of mutants. This suggests that the processes by which auxin acts on plant growth and development are complex and require the cooperation of different genes involved in auxin biosynthesis, transport, and signaling. In addition to the fact that genetic interaction among *YUCs* affects embryonic development, *YUCs* that genetically interact with *PID* contribute to cotyledon formation. The *yuc1 yuc4 pid-101* triple mutants displayed no cotyledon phenotype, which was absent in *yuc1 yuc4* double mutants or *pid-101* (Cheng, Dai, & Zhao, 2007). It seemed reasonable to assume that the PID-YUCs were involved in the same process to control the formation of the cotyledons.

3.1.2 | TAA

The TAA family in *Arabidopsis* consists of five members: *TAA1* (also known as *WEI8*), *TAR1*, *TAR2*, *TAR3*, and *TAR4*, which were divided

into two clades by phylo-genetic study. *TAA1*, *TAR1*, and *TAR2* are members of the same clade and are involved in IAA synthesis. They were found in different subcellular localizations, such as TAA1 localized in the cytoplasm and TAR2 localized in the ER membrane (Kriechbaumer et al., 2012; Matthes et al., 2019; Yu et al., 2016). The TAA gene family, like YUCs, is involved in several critical plant growth and development processes, such as embryogenesis, vascular patterning, and floral development (Cheng et al., 2006;Cheng, Dai, & Zhao, 2007; Stepanova et al., 2008; Won et al., 2011). It is not unexpected that the inactivation of *YUCs* or *TAAs* displays similar developmental defects since they are on the same pathway to produce IAA. For example, both *yuc1 yuc4 yuc10 yuc11* quadruple mutants and *wei8 tar1 tar2* triple mutants displayed no root or hypocotyl defects like the *arf5* mutant, which were also observed in *yuc1 yuc4 wei8 tar2* quadruple mutants (Cheng, Dai, & Zhao, 2007; Stepanova et al., 2008; Won et al., 2011). It was evident that *YUCs* and *TAAs* had a similar effect on the development of embryonic basal parts. The *wei8 tar2* double mutants combined with *pid-101*, as well as *yuc1 yuc4 pid-101* triple mutants, showed no cotyledon phenotype (Cheng, Qin, et al., 2007; Won et al., 2011). It was suggested that PID-TAA-YUCs were in the same pathway to regulate cotyledon formation. The genetic results show that the TAA and YUC-catalyzed IAA synthesis route is important for embryonic development processes, including cotyledon formation and hypophysis development.

3.2 | Auxin polar transport-related genes

3.2.1 | PIN1

Plant cell division and differentiation require the establishment and maintenance of auxin gradients determined by the polar transport of auxin, which is mediated by the different types of auxin transport carriers. Carriers, directing intercellular or intracellular auxin flow, mainly contain the PIN protein family, the ABC-type transporter PGP (P-GLYCOPROTEIN), and AUX/LAX (AUXIN1/LIKE-AUX1) protein (Friml et al., 2004; Geisler et al., 2005; Mravec et al., 2009; Terasaka et al., 2005; Yang et al., 2006). PIN1 is a protein that immediately comes to mind when discussing auxin transport due to its importance in auxin effluxion and the severe floral phenotype caused by the mutation in *PIN1*. The abnormal cotyledon phenotype and pin-like inflorescence phenotype caused by the *pin1* mutant are similar to those of the *pid* mutant (Bennett et al., 1995; Furutani et al., 2004). Based on the similarity of the cotyledon defect phenotypes in both the *pid* and *pin1* single mutants at the seedling stage, similar subcellular localization, and physical interaction between the two proteins, *PIN1* and *PID* may be in the same pathway that affects the number and symmetric pattern of cotyledons.

PIN1 belongs to the PIN protein family, which consists of eight members named PIN1–PIN8 and is involved in a variety of growth and development activities in *Arabidopsis* (Friml, 2010). The eight members can be generally divided into two types based on their protein motifs: canonical or noncanonical PINs. Compared with noncanonical PINs such as PIN5, PIN6, and PIN8, the canonical PINs, including PIN1–4 and PIN7, typically process longer, more highly conserved hydrophilic loops containing phosphorylation sites (Bennett et al., 2014). In addition to structural differences, the two types of proteins differ in subcellular localization and auxin transport activity. For example, PIN8, without phosphorylation motifs, is located in the endoplasmic reticulum and constitutively pumps out auxin independent of PID (Ung et al., 2022). Compared with PIN8, the polar localization in the plasma membrane and the exporting auxin activity of PIN1 is influenced by its phosphorylated state dependent on PID (Dai et al., 2012; Friml et al., 2004; Michniewicz et al., 2007; Ung et al., 2022; Zhang et al., 2010). The multiple mutants, like *pin1 3 4 7* quadruple mutants, displayed a more severe embryo phenotype, such as failure to develop any cotyledons with a certain percentage, than *pin1* (Vieten et al., 2005). PIN1 had a redundant effect on cotyledon development with other canonical PINs like PID and its orthologs. The regulatable shift in the apical-basal localization of the PIN protein on the plasma membrane mediated by PID or PP2A contributes to auxin gradient maintenance. Auxin at a certain concentration, which is partially maintained by PIN or PID proteins in cells at the symmetrical edge of the apical region of the transition embryo, appeared to be an initial signal for the formation of cotyledons. The *pin1 pid-2* double mutants had a high percentage of no cotyledon phenotype (approximately 47.3%) (Furutani et al., 2004). It was clear that PID and PIN1 were functionally redundant and could be involved in the same process that is crucial for cotyledon formation. Considering that no cotyledon phenotype was absent in the *pin1* or *pid-2* single mutant and that the phenotype was not fully penetrant in *pin1 pid-2*, PIN1 protein deficiency or abnormal distribution of PIN1 protein in the membrane is not the only factor that causes no cotyledon in *pin1 pid-2* double mutants. It was very likely that, in addition to sufficient PIN1 protein abundance, normal PIN1 function and distribution, other canonical PINs or another PID-regulated pathway, were required for cotyledon formation.

The *pin1 yuc1 yuc4* triple mutants failed to develop any true leaves and had normal cotyledons, while the *yuc1 yuc4 pid-101* triple mutants failed to develop cotyledons and still had a true leaf structure (Cheng, Qin, et al., 2007,c). Genetic interactions among PIN1, PID, and YUCs revealed that auxin-mediated cotyledon and true leaf formation processes were different and required different genes to initiate them. This meant that the PID-PIN pathway and the PID-YUCs pathway for cotyledon formation might be parallel. In the process of cotyledon formation, PID could potentially perform multiple functions rather than just phosphorylating PIN proteins.

3.2.2 | NPY1

The pin-like phenotype, characterized by severe inflorescence defects, has previously been found only in auxin-related mutants such as *pid*, *pin1*, and *arf5*, which have been implicated in auxin polar transport and signaling in *Arabidopsis*. In the case of the loss of function of undefined genes that gave rise to pin-like inflorescence, there was a

strong suggestion that the genes may be involved in auxin-correlative developmental progress. A pin-like inflorescence was developed when the ENP (ENHANCER OF PINOID) gene was disrupted in the background of yuc1 yuc4 double mutants, revealing a potential property of the ENP related to auxin. The enp mutant was also known as npy1 (naked pins in yuc mutants 1) (Cheng, Qin, et al., 2007). NPY1 encodes a protein mainly consisting of an N-terminal bric-a-brac, tramtrack, broad complex domain, and a C-terminal plant-specific NPH3 (NON-PHOTOTROPIC HYPOCOTYL 3) domain. NPY1 has 31 homologs in Arabidopsis (Cheng, Qin, et al., 2007; Glanc et al., 2021). The combination of mutations in NPY1 and its closest homolog, NPY5, also generated a pin-like phenotype (Cheng et al., 2008; Cheng, Qin, et al., 2007). The inflorescence defect phenotype in npy1 npy5, which was similar to pin1 and pid mutants, provided a clue that NPY may be involved in auxin polar transport. The npy1 npy3 or npy1 npy5 double mutants showed severe cotyledon defects, and npy1 npy2 npy3 npy4 npy5 quintuple mutants exhibit severe gravitropic characteristics (Furutani et al., 2011; Li et al., 2011). NPY1, together with its homologs, was indicated to redundantly regulate cotyledon and floral development, as well as gravitropism. During embryogenesis, NPY1 expression started at the globular stage and was restricted to cotyledon primordia in the early heart stage (Cheng, Qin, et al., 2007). NPY1 was involved in auxin-regulated cotyledon formation, which was further confirmed by a genetic interaction. A combination between npy1 and pid-101 mutants displayed no cotyledon phenotype, which was fully penetrant in double mutants and was not present in the npy1 mutant, despite a small percentage of abnormally fused cotyledons in the npy1 mutant (Cheng, Qin, et al., 2007). The interaction between NPY1 and AGC kinases, including PID, promoted PIN phosphorylation, which further enhanced NPY1 recruitment to the plasma membrane via positive feedback (Glanc et al., 2021). NPY1 and PIN1 may share the same pathway, similar to the relationship between PID and PIN1 in the formation of cotyledons. It is possible that the npy1 pin1 double mutants also fail to develop any cotyledon. The PID and NPY1 mediated pathway could be similar to that of PHOT1 and NPH3, which were homologs of PID and NPY1, respectively. PHOT1-NPH3 formed a complex to regulate phototropic responses (Motchoulski & Liscum, 1999; Pedmale & Liscum, 2007). Another pathway, mediated by the NPY1-PID-PIN1 complex, may be responsible for the initiation of cotyledon development.

3.2.3 | VPS28A

The delivery of the PIN1 protein within the cell for degradation or recycling was involved in various routes and different proteins containing components of ARF (ADP ribosylation factor, ARF), GEFs (guanine nucleotide exchange factor), and ESCRT (endosomal sorting complex required for transport), except for the localization of PIN1 on the plasma membrane, which was affected by PID or PP2A (Dai et al., 2012; Friml et al., 2004; Geldner et al., 2003; Liu et al., 2020; Scheuring et al., 2011). In plants, the ESCRT machinery plays a crucial role in a variety of biological processes, including suppressing ABA

signaling, regulating cytokinesis, and embryonic development (Li et al., 2019; Spitzer et al., 2006; Wang et al., 2017; Yu et al., 2016). The VPS28 protein (VACUOLAR PROTEIN SORTING 28), a member of the ESCRT complex, was responsible for auxin accumulation, and the specific expression pattern and proper localization of PIN1 (Liu et al., 2020). In Arabidopsis, VPS28 has two copies named VPS28A and VPS28B (Otegui, 2018). The subcellular localization of VPS28A and VPS28B was detected in the trans-Golgi network/early endosome and the post-Golgi apparatus/endosome (Liu et al., 2020; Scheuring et al., 2011), and two proteins were expressed throughout the embryonic period. Both genes are essential for embryonic development because simultaneous inactivation of the genes led to aborted embryo phenotype, which was absent in any single mutant (Liu et al., 2020). A homozygous point mutation in VPS28A under pid-101 background led to the no cotyledon phenotype. However, the T-DNA insertion mutants of VPS28A combined with the pid-101 mutant did not have any cotyledon phenotype. The point mutant in VPS28A displayed multiple developmental defects that were absent in these T-DNA insertion mutants of VPS28A. It was suggested that the point mutant in VPS28A, which resulted in a conversion of glutamic acid to lysine at the C-terminal instead of a prestop codon, gained new features in developmental processes, including cotyledon formation. The acetylation of the acquired lysine in the altered VPS28A protein, which neutralizes its intrinsic positive charge, may have changed the interaction between VPS28A and other proteins, which could have affected the formation of the ESCRT-I complex. The conversion has weakened the physical interaction between VPS28A and VPS23A (Liu et al., 2020). It is likely that VPS28A physically interacts with VPS28B and that the dimer, rather than a single protein, is important for proper PIN1 location and embryogenesis. The point mutation in VPS28A decreased the interaction between VPS28A and VPS28B and generated an intermediate threshold of the interaction that partially interrupted the expression pattern and regular turnover of related proteins such as PIN1, which was sufficient to produce no cotyledon under the pid-101 background.

3.3 | Auxin response-related genes

3.3.1 | MAB2

MEDIATOR (MED) complex was closely correlated with transcriptional regulation by interacting with RNA polymerase II and transcription factors (Malik & Roeder, 2010). In Arabidopsis, two components of the MED complex, called MED12 and MED13, were involved in embryo patterning, postembryonic growth, and flowering (Gillmor et al., 2010, 2014). MED13, also known as MAB2 (MACCI-BOU 2), was found to play a key role in the formation of cotyledons with PID, as pid-2 mab2 double mutants resulted in no cotyledon phenotype (Ito et al., 2011). In the cotyledon primordia of the mab2 mutant, the expression and subcellular localization of PIN1 were consistent with those of WT, and the DR5 signals had a significant reduction compared to WT (Ito et al., 2011). It has been suggested that

MAB2 is not involved in auxin polar transport but may influence the expression of several auxin response genes that are essential for cotyledon formation in conjunction with PID function.

3.4 | Both auxin polar transport and response-related genes

3.4.1 | MOB1A

In *Arabidopsis*, a gene named *AtMOB1A* is a versatile modulator of auxin-mediated plant growth and development because *AtMOB1A* has genetically interacted with various auxin-related genes such as *YUC*, *PID*, and the *TIR1* gene family, which are representative in auxin biosynthesis, auxin polar transport, and auxin signaling, respectively (Cheng, Dai, & Zhao, 2007; Cui et al., 2016; Michniewicz et al., 2007; Tan et al., 2007). The various synergistic interactions involving *AtMOB1A* are important for embryogenesis, inflorescence development, and fertility (Cui et al., 2016). The homologs of AtMOB1A in mammals participate in a Hippo signaling pathway that affects cell fate and pattern formation. In *Arabidopsis*, AtMOB1A, which is localized in the nucleus, cytoplasm, and plasma membrane, has three other homologous genes named AtMOB1B, AtMOB1C, and AtMOB1D, respectively (Cui et al., 2016; Pinosa et al., 2013). AtMOB1A physically interacted with a few proteins to coregulate multiple plant biological processes. For example, AtMOB1A and AtMOB1B belonged to a common clade and interacted with each other to regulate plant development and jasmonate accumulation (Guo, Chen, et al., 2020). SIK1(serine/threonine kinase 1), which encoded a Ste20/Hippo-like protein, interacted with AtMOB1A to control organ size (Xiong et al., 2016). Two MAPKKK kinases, MAP 3 K ε1 and MAP 3 K ε2, and three AGC kinases, NDR2/4/5, which are all members of the AGC kinase family like PID, interacted with AtMOB1A or AtMOB1B to control pollen germination and development (Mei et al., 2022; Zhou et al., 2021). *AtMOB1A* was expressed throughout embryonic development, consistent with its role in embryogenesis, as demonstrated by the aberrant embryo phenotypes that occurred in *atmob1a* mutant from the 8-cell stage to the globular stage (Cui et al., 2016). Disruption of *AtMOB1A* enhances the *pid-101* phenotype, with up to 90% of homozygous *atmob1a pid-101* double mutants lacking cotyledons, whereas the *atmob1a* mutant had normal cotyledons. This suggested that AtMOB1A alone played a minor role in the development of cotyledons. DR5-GFP signals were decreased in *atmob1a*, and the localization pattern of PIN1-GFP signals was altered in *atmob1a pid-101* mutants (Cui et al., 2016). Given that AtMOB1A and PID had overlapped localizations on the plasma membrane and that Mats, the Drosophila homolog of MOB1, interacted with the AGC protein kinase-NDR and modified its protein activity to regulate the Hippo signaling pathway, AtMOB1A could bind to PID and alter PID activity to regulate the PIN1 localization pattern for the control of cotyledon formation. The mechanism could be similar to the relationship between hMOB1 and NDR (nuclear Dbf2-related kinases) in humans (Hergovich et al., 2005). However, the interaction test between them

is negative using common biochemical tools, and it still cannot rule out the possibility of two proteins interaction because the interaction could be short-lived and require a special technique such as proximity labeling to detect it, or the interaction could require some kind of signal to initiate it or another protein as a bridge to connect PID and AtMOB1A.

3.4.2 | MAB1

The mitochondrial pyruvate dehydrogenase complex (PDC), consisting of E1, E2 and E3 enzymes, affects the tricarboxylic acid cycle to produce ATP for cell activity (Mooney et al., 2002). The E1 enzyme contains a catalytic subunit α and a regulatory subunit β. Both subunits are closely associated with auxin functions in *Arabidopsis* (Ohbayashi et al., 2019). *IAR4*, which encodes an E1 α subunit, is involved in the transformation of indole-3-pyruvate into indole-3-acetyl-coenzyme A, which subsequently affects auxin homeostasis (LeClere et al., 2004). The *MAB1* gene (*MACCI-BOU 1*) encodes a mitochondria-localized E1 β subunit, which was effective for the developmental pattern of cotyledons, since *mab1* mutants displayed a single-cotyledon phenotype with a certain percentage of cotyledons. In the *mab1* mutant, the DR5 signal increased and the abundance of PIN1 decreased at the tip of the cotyledon primordia during the late embryonic development period. It indicated that the abundance of PIN1 was maintained in part by the MAB1-involved TCA cycle (Ohbayashi et al., 2019). The DR5 signal decreased in the *pin1* mutant and increased in the *pgp1 pgp19* (Mravec et al., 2008). It is possible that the increased DR5 signal was caused by decreased PGP1 and PGP19 levels resulting from the loss of function in *MAB1*. The increased auxin level in the *mab1* mutant may not be caused by a decreased PIN1 level but rather by levels of PGP1 and PGP19 that prevent auxin in cells at the tip of the cotyledon primordia from flowing into neighboring cells. The *mab1 pid-2* double mutants had no cotyledon phenotype that was not found in the *mab1* mutant. The increased auxin level in the *mab1 pid-2* background may not be a significant factor that causes the absence of the cotyledon phenotype. It can be inferred that that the formation of cotyledons requires not only appropriate auxin concentration and developmental signals but also energy to initiate it.

3.4.3 | SAC7

Phosphoinositides, derived from phosphatidylinositol, have been proven to be important for plant growth and development (Barbosa et al., 2016; Guo, Yue, et al., 2020). Different phosphoinositides such as phosphatidylinositol monophosphates, bisphosphates, or triphosphates can be interconverted through phosphorylation and dephosphorylation by kinases and phosphatases, respectively (Boss & Im, 2012; Meijer & Munnik, 2003). SUPPRESSOR OF ACTIN7 (SAC7), a member of the gene family encoding one type of SAC domain-containing protein (Zhong & Ye, 2003). The SAC domain processes

phosphoinositide phosphatase activities (Hughes et al., 2000). In *Arabidopsis*, the SAC family contains nine members, named SAC1-SAC9, which are generally divided into three clades. SAC6, SAC7, and SAC8 belong to the same clade and coregulate plant development (Song et al., 2021; Zhong & Ye, 2003). None of the single mutants of the three genes displayed obvious defects during every growth period of plants, except the *sac7* mutant had mild root hair defects (Song et al., 2021; Thole et al., 2008). The *sac6 sac7* and *sac7 sac8* double mutants and *sac6 sac7 sac8* triple mutants showed decreased expression of DR5 and serious developmental defects during embryonic development. The polarity of PIN1-GFP was altered in *sac7* mutant, *sac6 sac7*, and *sac7 sac8* double mutants. *SAC7* played an important role in the proper subcellular localization of PIN1 and was redundant with *SAC6* and *SAC8* in embryonic development. The level of PIP$_2$ (phosphatidylinositol-4,5-bisphosphate) increased in the *sac7* mutant. PIP$_2$ is a kind of phospholipid that regulates several biological functions. PIP$_2$ can be hydrolyzed into two types of second messengers, DAG (diacylglycerol) and IP$_3$ (Inositol-1,4,5-trisphosphate), which, respectively, mediates downstream signal transduction (Balakrishnan et al., 2015; Cocco et al., 2015). The proteins with a PH or BAR domain can bind to PIP$_2$, which recruits them to the plasma membrane, and that becomes an important prerequisite for activating their own or other protein functions. The increased PIP$_2$ level in *sac7* may alter the concentration of DAG or IP3 and downstream protein activity. The absence of cotyledon phenotype appearing in the *sac7 pid-101* double mutant shows that *SAC7* and *PID* together were important for cotyledon formation (Song et al., 2021). The function and activity of some important proteins such as protein kinases, ion channels, and DAG or IP$_3$-mediated phosphoinositide signaling, which were affected by the increased level of PIP$_2$ in *sac7*, could be related to the production of no cotyledon.

3.4.4 | ERfs

Erfs (ERECTA family) are a kind of RLKs (Receptor-like kinases), which play essential functions in the development and growth of plants (Morillo & Tax, 2006). ERfs contain three members named ER (ERECTA), ERL1 (ERECTA-LIKE1), and ERL2, which encode plasma membrane-specific leucine-rich repeat (LRR) receptor-like kinases involved in phototropism, initiation of leaf and cotyledon primordia, and auxin-regulated leaf formation (Chen et al., 2013; DeGennaro et al., 2022; Tameshige et al., 2016). *Erfs* were involved in auxin transport and metabolism but not in direct regulation of auxin response (DeGennaro et al., 2022). In the *er erl1 erl2* triple mutant, the distribution of auxin was altered and PIN1 expression increased in the cytoplasm. The majority of *er erl1 erl2 pid-3* quadruple mutants displayed no cotyledon phenotype that was absent in *er erl1 erl2* triple mutants. Auxin concentration increased in the L1 layer at the periphery of the SAM (shoot apical meristem), where the cotyledon primordia is supposed to initialize in the *er erl1 erl2 pid-3* mutant. An increased level of auxin was indicated to have little correlation with no cotyledon production in *er erl1 erl2 pid-3* mutants. Based on the abnormal

phenotype with aberrant cotyledon numbers that appear in both *pid* and *er erl1 erl2* and their similar subcellular localization and protein properties between PID and ERfs, it is hypothesized that Erfs receive some signals and activate their protein kinase activity, which promotes Erfs to physically interact with PID and phosphorylate PID to activate a novel auxin-independent signaling pathway to initiate cotyledon formation.

4 | CONCLUSIONS AND PERSPECTIVES

Among the numerous auxin-related genes in *Arabidopsis*, *PID* is a special gene as it genetically interacts with multiple types of genes to control the formation of cotyledons. All of these genes are involved in auxin synthesis, polar auxin transport, or auxin response, which overlaps in part with the function of PID in cotyledon formation. The vast majority of genes belong to different categories and have various functions. It is impossible that all these genes, including *PID*, are on the same pathway to regulate cotyledon formation. It is indicated that *PID*-mediated cotyledon formation is a multibranched process. The concentration, activity, or distribution of PIN1 have been altered in most mutants, proving that the PIN1-mediated auxin gradient is very critical for the formation of cotyledons. The no cotyledon phenotype presenting in the *yuc1 yuc4 pid-101* triple mutants indicated that an appropriate auxin concentration is required for the formation of cotyledons. An adequate amount of auxin may alter the expression of auxin-responsive genes through auxin signaling or as a developmental signal to activate plasma membrane-localized protein kinases, which are necessary for the initiation of cotyledon formation.

It is clear that *PID*-mediated auxin action is very crucial for cotyledon formation. On the basis of these genetic interactions, a *PID*-centered network can be established. However, PID is not only an essential factor in determining cotyledon formation, as *pin1 supo1* double mutants also displayed no cotyledon phenotype. *SUPO1* (suppressors of PIN1 overexpression 1) encodes an inositol polyphosphate 1-phosphatase. The concentration of inositol trisphosphate and Ca^{2+} increased in the *supo1* mutant (Zhang et al., 2011). Given the increased level of PIP$_2$ in the *sac7* mutant and no cotyledon phenotype in *pin1 supo1* and *sac7 pid*, it is a hint that phospholipid or Ca^{2+}-involved signaling is also important for cotyledon formation. Although no cotyledon appeared in *arf5 iaa12*, *arf5 arf7*, and *mab2 iaa12*, these double mutants also had other severe developmental phenotypes such as no root and small structure (Hardtke & Berleth, 1998; Ito et al., 2011). It was suggested that the development of cotyledon primordium cells mediated by PID at the transition stage was specific and played a minor role in the development of other regional cells, such as hypophysis cells, during embryogenesis. Although there have been many types of gene-synergistic interactions to regulate cotyledon production, there may not be a comprehensive genetic network because there are no genetic data between PID and canonical auxin signaling genes such as *TIR1* family genes, *ARFs*, and *IAA* in the nucleus. The *tir1 afb1 afb2 afb3* quadruple mutants and the *arf5* or *iaa12* single mutant also displayed cotyledon defects. It is quite likely

that PID combined with these genes is also essential for cotyledon formation.

AUTHOR CONTRIBUTIONS

CONFLICT OF INTEREST STATEMENT

DATA AVAILABILITY STATEMENT

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SUPPORTING INFORMATION

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