mRNA surveillance complex PELOTA–HBS1 regulates phosphoinositide-dependent protein kinase1 and plant growth

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Abstract

The quality control system for messenger RNA (mRNA) is fundamental for cellular activities in eukaryotes. To elucidate the molecular mechanism of 3’-phosphoinositide-dependent protein kinase1 (PDK1), a master regulator that is essential throughout eukaryotic growth and development, we employed a forward genetic approach to screen for suppressors of the loss-of-function T-DNA insertion double mutant pdk1.1 pdk1.2 in Arabidopsis thaliana. Notably, the severe growth attenuation of pdk1.1 pdk1.2 was rescued by sop21 (suppressor of pdk1.1 pdk1.2), which harbors a loss-of-function mutation in PELOTA1 (PEL1). PEL1 is a homolog of mammalian PELOTA and yeast (Saccharomyces cerevisiae) DOM34p, which each form a heterodimeric complex with the GTPase HBS1 (HSP70 SUBFAMILY B SUPPRESSOR1, also called SUPERKILLER PROTEIN7, SKI7), a protein that is responsible for ribosomal rescue and thereby assures the quality and fidelity of mRNA molecules during translation. Genetic analysis further revealed that a dysfunctional PEL1–HBS1 complex failed to degrade the T-DNA-disrupted PDK1 transcripts, which were truncated but functional, and thus rescued the growth and developmental defects of pdk1.1 pdk1.2. Our studies demonstrated the functionality of a homologous PELOTA–HBS1 complex and identified its essential regulatory role in plants, providing insights into the mechanism of mRNA quality control.
Introduction

Living organisms need to monitor both the quantity and the quality of biomolecules, such as nucleic acids and proteins, to accomplish various life activities. Protein quality control is ensured by many levels of regulation for which messenger RNA (mRNA) quality is essential for biosynthesis of the correct corresponding protein: transcriptional, post-transcriptional, translational, and post-translational. The quality and fidelity of mRNAs are monitored by cells autonomously, and aberrant mRNAs need to be recognized by intrinsic molecular machineries to release stalled ribosomes and get degraded themselves (Tsuboi et al., 2012). According to current understandings, there are at least three different mRNA surveillance mechanisms: nonsense-mediated decay (NMD), no-stop decay (NSD), and no-go decay (NGD). NMD and NSD target mRNAs with premature stop codons (terminated too soon) and lacking stop codons (failing to terminate), respectively. NGD targets mRNAs stalled during translational elongation, e.g. those mRNAs with secondary structures or modifications. In mammals and yeasts, the PELOTA–HBS1 (Dom34p-HBS1p in yeasts) complex plays an essential role in regulating ribosomal rescue by recognizing the stalled ribosomes to initiate their recycling; this complex has been reported as important for all NMD, NSD, and NGD mechanisms (Shoemaker et al., 2010; Hilal et al., 2016). However, little was known about the mRNA quality control in plants so far.

3'-Phosphoinositide-dependent protein kinase1 (PDK1) is conserved in eukaryotes and plays important roles in regulating growth and development in various organisms (Pearce et al., 2010). As a key member of the cAMP-dependent protein kinase A/protein kinase C (AGC) kinase family (Alessi, 2001), PDK1 is important for the activation of many AGC kinases and other substrates/regulators. Studies have revealed that PDK1 plays crucial roles in the signaling pathways activated by growth factors and hormones, sustains and regulates the balance among cell growth, division, and apoptosis in mammals (Alessi, 2001; Storz and Toker, 2002; Mora et al., 2004), and thus is critical for growth and development. However, loss-of-function mutation of PDK1 is lethal in various species, such as yeasts (Saccharomyces cerevisiae; Casamayor et al., 1999; Inagaki et al., 1999), fruit flies (Drosophila melanogaster; Rintelen et al., 2001), and mice (Mus musculus; Lawlor et al., 2002), which makes it challenging to study the downstream regulation, and the functional mechanism of PDK1 is still not completely understood.

Differently from mammals, loss-of-function or knockout pdk1 mutant plants are viable, despite exhibiting severe developmental defects, as observed in rice (Matsui et al., 2010), Arabidopsis (Camehl et al., 2011; Tan et al., 2020; Xiao and Offringa, 2020), and moss (Dittrich and Devarenne, 2012). Therefore, plant models provide a more attractive approach to further identify the genetic interactors of PDK1. There are two PDK1 paralogs in Arabidopsis, PDK1.1 and PDK1.2 (also called PDK1 and PDK2), which have redundant functions (Dittrich and Devarenne, 2012; Tan et al., 2020; Xiao and Offringa, 2020). PDK1 binds to phospholipids, which regulates its activity as well as its subcellular localization (Deak et al., 1999; Anthony et al., 2004; Tan et al., 2020). As a master regulator of the AGC family, PDK1 was proposed to participate in various growth and developmental processes by phosphorylating distinct kinase substrates (Zegzouti et al., 2006b; Tan et al., 2020, 2021; Xiao and Offringa, 2020). For example, PDK1.1 regulates root hair development through phosphorylating OXIDATIVE SIGNAL INDUCIBLE1 (OXI1)/AGC2-1 kinase (Anthony et al., 2004; Rentel et al., 2004). PDK1 binds to phospholipids, which phosphorylates PINOID (PIN), an essential regulator of PIN-FORMED (PIN) auxin efflux carriers, was phosphorylated by PDK1 and thus activated in vitro (Friml et al., 2004; Zegzouti et al., 2006a). Notably, the pdk1.1 pdk1.2 double mutants uncovered the important role of plant PDK1. Both PDK1.1 and PDK1.2 are expressed in vascular tissues and show a predominant localization at the basal side of cell plasma membrane (PM) as well as at cytoplasm in root stele (Tan et al., 2020; Xiao and Offringa, 2020). Importantly, the basal localization of PDK1 dominates the role of AGC kinase substrates with the same subcellular distribution, including D6 Protein Kinase (D6PK)/D6 Protein Kinase Likes (D6PKLs; Zourelidou et al., 2009) and PROTEIN KINASE-ASSOCIATED WITH BRX (PAX; Marhava et al., 2018), to jointly participate in the regulation of polar auxin transport (Tan et al., 2020, 2021; Xiao and Offringa, 2020).

To further identify regulators involved in the PDK1 pathway, a forward genetic approach was employed. Using an ethyl methanesulfonate (EMS) population of Arabidopsis pdk1.1 pdk1.2 double mutant that displayed severe growth defects, a suppressor screening was performed. In this study, characterization of an identified mutant, sop21 (suppressor of pdk1.1 pdk1.2), reveals that deficiency of the translational mRNA surveillance complex PELOTA–HBS1 rescues the defective phenotype of pdk1.1 pdk1.2. Our studies demonstrate the functionality of a homologous PELOTA–HBS1 complex in plants and provide informative clues on the control of mRNA surveillance and thus protein homeostasis.

Results

Deficiency of PEL1 suppresses the defective growth of Arabidopsis pdk1.1 pdk1.2

Two paralogous PDK1 genes in Arabidopsis thaliana, PDK1.1 (AT5G04510) and PDK1.2 (AT3G10540; Camehl et al., 2011; Dittrich and Devarenne, 2012; Tan et al., 2020), exhibit overlapping and widespread expression patterns in various tissues, as revealed by quantitative polymerase chain reaction (PCR) and GUS staining analyses (Supplemental Figure S1).
Notably, both PDK1.1 and PDK1.2 were detected to be expressed in seedlings, roots, rosette leaves, cauline leaves, stems, flowers, and siliques, with strong expression in vascular tissues (Supplemental Figure S1), similar as previously reported (Tan et al., 2020). The pdk1 single loss-of-function mutants, pdk1.1-2 and pdk1.2-4 (hereafter as pdk1.1 and pdk1.2, respectively), displayed no obvious growth phenotype (Tan et al., 2020), whereas the double mutant pdk1.1 pdk1.2 exhibits a range of developmental defects (Figure 1; Supplemental Figure S2), including suppressed growth (smaller leaves and shorter siliques), reduced axillary shoots, suppressed primary root elongation and lateral root initiation, substantially decreased fertility, and abnormal floral development (Tan et al., 2020). This is consistent with the crucial roles of PDK1 in other organisms and confirms the essential role of PDK1 in regulating plant growth and development. Several aspects of those developmental defects can be explained by known AGC kinases, such as D6PK (Tan et al., 2020), PAX (Xiao and Offringa, 2020), and AGC1.5/7 (Zhang et al., 2009; Xiao and Offringa, 2020). However, whether there are additional components in the PDK1 pathway, other than AGC kinases, remains unclear.

To elucidate the underlying mechanism of PDK1 function, a forward genetic screen was performed. Seeds of pdk1.1 pdk1.2 were used to generate a mutant population by EMS mutagenesis, and suppressors of pdk1.1 pdk1.2 (sop21, suppressor of pdk1.1 pdk1.2) were screened based on rescued growth (Page and Grossniklaus, 2002). More than 10 suppressors were obtained from a M$_2$ population of approximately 80,000 plants, and a recessive mutant, sop21, that showed obviously rescued growth of pdk1.1 pdk1.2 (Figure 1; Supplemental Figure S2), was characterized first. Compared to the significantly suppressed growth of pdk1.1 pdk1.2 adult plants, the rosette size of pdk1.1 pdk1.2 sop21 is comparable to that of wild-type (WT; Figure 1A; Supplemental Figure S2D). Similar degree of rescue was also observed for the fertility, silique length, inflorescence morphology, and floral development (Figure 1, B and C; Supplemental Figure S2, E and F). Notably, the lateral root numbers were only partially rescued to ~50% of WT, though with a complete rescue of primary root growth (Figure 1, D–F).

To identify the causative mutation in sop21, 102 progenies (referred as BC$_2$F$_2$) showing rescue phenotypes were selected from a segregating pool of backcross F$_2$ individuals (102 rescued phenotype: 296 pdk1.1 pdk1.2 phenotype, for 1:3 ratio [$\chi^2 = 0.084, P > 0.75$; Chi-square test], which indicated a causal single recessive mutation) and used for DNA extraction and subsequent deep sequencing (Allen et al., 2013). Systemic analysis revealed that sop21 carried a mutation in PEL1 (AT4G27650; Csorba et al., 2018), which led to an early stop at amino acid residue 27 (tryptophan to terminator, W27*), resulting in the translationally premature termination (Supplemental Figure S2A). Reverse transcription quantitative real-time PCR (RT-qPCR) and GUS staining analyses revealed that the PEL1 gene was expressed in seedlings, roots, rosette leaves, cauline leaves, stems, flowers, and siliques, with elevated expression in vascular tissues (Supplemental Figure S3), which is similar to the expression of PDK1s. Cross of a null T-DNA insertion allele pel1 (SALK_124403, also named lesion mimic leaf1-1, lml1-1; Qin et al., 2018; Supplemental Figure S2, B and C) with pdk1.1 pdk1.2 also suppressed the growth defects of pdk1.1 pdk1.2 at various aspects (Figure 1), verifying that the suppression of pdk1.1 pdk1.2 phenotype in sop21 was a result of PEL1 deficiency. Though pel1 mutant was previously shown to exhibit a reduced growth rate (Qin et al., 2018), both sop21 and pel1 mutants grew normally in our hands, perhaps due to different growth conditions. In addition, the expression of PEL1–FLAG driven by a CaMV35S promoter in sop21 restored the pdk1.1 pdk1.2 phenotype (Supplemental Figure S4, A and B), confirming that PEL1 deficiency suppressed the growth defects of pdk1.1 pdk1.2. The overexpression of PEL1–FLAG driven by CaMV35S in WT background did not exhibit any obvious phenotype (Supplemental Figure S4, C and D).

Next, we constructed 35S::PEL1–GREEN FLUORESCENT PROTEIN (GFP) and 35S::mCherry–PEL1 transgenic plants to study the subcellular localization of PEL1. We observed the presence of PEL1–GFP and mCherry–PEL1 in the cytoplasm and nucleus (Figure 2A). The subcellular localization of PEL1 partially overlapped with that of PDK1.1, which also showed residence in cytoplasm, but PEL1–GFP did not share the PM localization with PDK1.1 (Figure 2A). Further analysis using tobacco leaves revealed that both mCherry–PEL1 (Figure 2B) and mCherry–PDK1.1 (Figure 2C) did not distribute equally in cytoplasm, and indeed they exhibited a similar feature in that both proteins localized to certain compartments associated with endoplasmic reticulum (ER).

Presence of the mRNA surveillance complex PELOTA–HBS1 in Arabidopsis

Most of our knowledge about the PELOTA–HBS1 complex function comes from the studies in animals and yeast. Translation of aberrant mRNAs leads to stalling of translational machinery, and arrested ribosomes are specifically recognized by the PELOTA–HBS1 complex to initiate their recycling (Shoemaker et al., 2010; Pisareva et al., 2011; Tsuboi et al., 2012; Saito et al., 2013). Mammalian and yeast PELOTA/DOM34p interact with the HBS1 GTPase, a translation elongation factor EF1A/initiation factor IF2 family protein, to form a heterodimer and bind to stalled ribosomes, ultimately leading to ribosome rescue. Furthermore, HBS1 is involved in post-transcriptional gene silencing (Csorba et al., 2018; Lange et al., 2019). Homology analysis in Arabidopsis genome by using yeast and human HBS1 identified two candidate HBS1 homologs, AT5G16030 and AT1G18070. Sequence alignment analysis showed that only AT5G16030 had the conserved HBS1_C domain, indicating that AT5G16030 (designated as AthHBS1, HBS1) is the HBS1 homolog in Arabidopsis (Supplemental Figure S5). As reported previously, AT1G18070 is a close homolog of eukaryotic release factor 3, which terminates translation and releases...
Figure 1 The sop21 mutation or deficiency of PEL1 or HBS1 restores the growth defects of Arabidopsis pdk1.1 pdk1.2 double mutant. A, The sop21 mutation or loss of function of PEL1 or HBS1 suppresses the growth defect of pdk1.1 pdk1.2, including the reduced growth and delayed bolting. 3- or 5-week-old Col-0, pdk1.1 pdk1.2, pdk1.1 pdk1.2 pel1, pdk1.1 pdk1.2 sop21 (pdk1.1 pdk1.2 pel1W27), pdk1.1 pdk1.2 hbs1 and various mutant plants are shown. Scale bars, 5 cm. B, Siliques and setting rate of Col-0 and various mutants. Scale bars, 2 cm (upper) or 1 mm (lower). Defective/abnormal seeds of pdk1.1 pdk1.2 are highlighted with red arrowheads. C, Quantification of the silique lengths of Col-0 and various
ribsomes (Brunkard and Baker, 2018). Considering that HBS1 functions in the same pathway as PEL1 in Arabidopsis (Csorba et al., 2018), it was then chosen for further investigations. Indeed, analyses of yeast two-hybrid (Figure 3A), bimolecular fluorescence complementation (BiFC; Figure 3B) and GST pull-down assays (Figure 3C) revealed the PEL1–HBS1 interactions both in vivo and in vitro, confirming the presence of a homologous PELOTA–HBS1 complex in plants. In addition, a recent study in plants characterizing the roles of PELOTA and HBS1 in nonstop mRNA decay (Csorba et al., 2018) further supports the presence of a functional PELOTA–HBS1 complex in Arabidopsis. However, whether the PELOTA–HBS1 complex participates in other types of mRNA surveillance mechanisms requires further investigations.

Similarly to PEL1 and PDK1s, the HBS1 gene was ubiquitously expressed (Supplemental Figure S6A) and a knock-down mutant of HBS1, hbs1 (Supplemental Figure S6, B–D) also suppressed the pdk1.1 pdk1.2 phenotypes (Figure 1, A and B), indicating that deficiency of the translational mRNA surveillance complex PELOTA–HBS1 led to the suppression of pdk1.1 pdk1.2 phenotypes. These observations physically and functionally support the existence of PELOTA–HBS1 complex.

PELOTA–HBS complex regulates the degradation of aberrant PDK1 transcripts

The pdk1.1-2 and pdk1.2-4 alleles we used contain T-DNA insertions close to their 3′-ends. It has been speculated by Xiao and Offringa (2020) that such alleles are likely to lead to the production of a functional truncated protein. Therefore, in the same publication, this was proposed to explain the lack of strong phenotypes for double mutant combinations pdk1.1-2 pdk1.2-2 and pdk1.1-2 pdk1.2-3 observed in previous studies ( Camehl et al., 2011; Scholz et al., 2019). However, it is unlikely that a partially functional truncated protein could explain the previous failure to observe the obvious defects in these alleles, since recent studies by Tan et al. (2020) and Xiao and Offringa (2020) both reported highly similar phenotypes for different allele combinations. Further comparisons confirmed that the pdk1.1 pdk1.2 (pdk1.1-2 pdk1.2-4) alleles used here in our study, as well as double mutants generated with pdk1.1-2 and other pdk1.2 T-DNA alleles, recapitulated the phenotype of double mutant combinations with the CRISPR alleles pdk1.1-13 or pdk1.1-14 reported by Xiao and Offringa (2020; Supplemental Figure S7, A and B). Therefore, we speculate that previous failures to observe strong growth and development defects in pdk1.1-2 pdk1.2-2 or pdk1.1-2 pdk1.2-3 double mutant lines ( Camehl et al., 2011; Scholz et al., 2019) are most likely due to a failure to achieve true double homozygous plants.

Interestingly, we also noted that the rosette size of pdk1.1 pdk1.2 plants is larger than other double mutant combinations, suggesting the remaining function of PDK1s (Supplemental Figure S8). RT-qPCR analysis revealed that there was no detectable expression of either PDK1.1 or PDK1.2 in pdk1.1 pdk1.2 backgrounds, with the primers amplifying regions across T-DNA insertions (Figure 4, A and B), ruling out the possibility of existing full-length PDK1.1 or PDK1.2 transcripts. Nonetheless, when primers amplified the fragments before T-DNA insertions, we detected increased levels of truncated transcripts of PDK1.1 and PDK1.2 (to ~50% or 90% of Col-0, respectively) in pdk1.1 pdk1.2 sop21, pdk1.1 pdk1.2 pel1, and pdk1.1 pdk1.2 hbs1, compared to those in pdk1.1 pdk1.2 (10% and 25% of Col-0 for PDK1.1 and PDK1.2, respectively; Figure 4, C and D). The decrease of PDK1.1 and PDK1.2 transcripts was further confirmed by semi-quantitative PCR analysis (Supplemental Figure S8A). The PELOTA–HBS1 complex is responsible for the release of arrested ribosomes during the translation of aberrant mRNAs, so called “mRNA surveillance”, and these mRNAs are degraded (Csorba et al., 2018). T-DNA insertions at the 3′-ends of PDK1.1 and PDK1.2 coding regions might lead to aberrant PDK1.1 and PDK1.2 transcripts fused with a partial T-DNA fragment, and these mRNAs could be degraded by the mRNA surveillance mechanisms. Therefore, our observation that only 10% and 25% of PDK1.1 and PDK1.2 5′-fragments were detected in pdk1.1 pdk1.2 confirms the instability of these transcripts in planta. Notably, there was an increase of their expressions in pdk1.1 pdk1.2 sop21, pdk1.1 pdk1.2 pel1, or pdk1.1 pdk1.2 hbs1 plants. The above observations suggested that the decay of aberrant PDK1.1 and PDK1.2 transcripts is mediated by the PEL1–HBS1-mediated mRNA surveillance mechanism. This led us to test whether the increased levels of truncated PDK1.1 or PDK1.2 could explain the rescue of pdk1.1 pdk1.2.

By transforming an mCherry-fused PDK1.1N (1–399 aa; Tan et al., 2020) driven by pPDK1.1 promoter into pdk1.1 pdk1.2, a partial rescue was observed (Figure 4E). In addition, the overexpression of Venus-PDK1.1N or Venus-PDK1.2N (encoding 1–394 aa) driven by a CaMV35S promoter

**Data are presented as means ± so (n > 30). n = 37, 36, 17, 54, and 51 siliques for Col-0, pdk1.1 pdk1.2, pel1, pdk1.1 pdk1.2 pel1, and pdk1.1 pdk1.2 sop21, respectively. Different letters represent significant differences, P < 0.05. Corrected P-values were calculated by one-way ANOVA with a Tukey’s multiple comparison test. D. Root elongation and lateral root formation of Col-0 and various mutants. Two-week-old seedlings were observed. Scale bar, 1 cm. Representative images are shown. E. Lengths of primary roots of 2-week-old Col-0 and various mutants were calculated. Data are presented as means ± so (n > 30), n = 56, 65, 52, 62, and 30 plants for Col-0, pdk1.1 pdk1.2, pel1, pdk1.1 pdk1.2 pel1, and pdk1.1 pdk1.2 sop21, respectively. Different letters represent significant differences, P < 0.05. Corrected P-values were calculated by one-way ANOVA with a Tukey’s multiple comparison test. F. Numbers of emerged lateral roots of 2-week-old Col-0 and various mutants were counted. Data are presented as means ± so (n > 30), n = 61, 65, 63, 63, and 52 plants for Col-0, pdk1.1 pdk1.2, pel1, pdk1.1 pdk1.2 pel1, and pdk1.1 pdk1.2 sop21, respectively. Different letters represent significant differences, P < 0.01. Corrected P-values were calculated by one-way ANOVA with a Tukey’s multiple comparison test.
Figure 2 Subcellular localizations of PEL1 and PDK1.1. A, Stable transgenic lines revealed that PEL1 localized to the cytoplasm and nucleus, and that PDK1.1-GFP and PDK1.2-GFP resided at PM and cytoplasm. Five-day-old 35S::PEL1-GFP, 35S::mCherry-PEL1, 35S::PDK1.1-GFP, and 35S::PDK1.2-GFP T2 seedlings were observed by CLSM. The “Green Fire Blue” LUT was used for GFP, and “mpl-inferno” LUT was used for mCherry visualizations, based on fluorescence intensity (as indicated by the color scale) by Fiji. Lower panels are enlarged view of the squared region of the upper panels. Scale bars, 20 μm. At least three independent lines were obtained for each genotype, and similar localizations were observed. Representative images are shown. n = 7, 8, 10, 12 individual plants for 35S::PEL1-GFP, 35S::mCherry-PEL1, 35S::PDK1.1-GFP, and 35S::PDK1.2-GFP, respectively. B–E, Fluorescence observations showed that both PEL1 and PDK1.1 localized to certain cytoplasm compartments associated with the ER. Fusion proteins mCherry-PEL1 (B and C) and mCherry-PDK1.1 (D and E) were transiently expressed with ER-specific GFP-HDEL proteins in tobacco leaves. Samples were observed 48 h after infiltration. Scale bars, 20 μm. Lower parts are enlarged view of the squared region of the upper parts. The position for quantification (right) was indicated with dashed lines across the images. Each pair of transformations was performed with five leaves and repeated twice. n = 10 and 11 samples for mCherry-PEL1+GFP-HDEL and mCherry-PDK1.1+GFP-HDEL, respectively.
completely rescued the phenotype of pdk1.1 pdk1.2 (Supplemental Figure S8B). Therefore, we conclude that the pel1 and hbs1 mutations might rescue the phenotype of pdk1.1 pdk1.2 via disrupting the function of PELOTA–HBS1 mRNA surveillance complex, and thus upregulating the amount of N-terminal truncated proteins of PDK1.1 and PDK1.2, which preserve functional kinase activity (Xiao and Offringa, 2020). Further genetic analysis on these double mutants by Xiao and Offringa (2020) and pel1 might help to confirm whether these effects are allele-specific.

PDK1 regulates development and stress responses through coordinating multiple metabolic pathways

The PELOTA–HBS1 complex regulates the mRNA quality control by rescuing stalled ribosomes during protein biosynthesis (Csorba et al., 2018). PDK1 is also an essential regulator of protein translation, via modulating the activity of ribosome RPS6 proteins through S6K AGC kinase (Mahfouz et al., 2006; Pearce et al., 2010; Kim et al., 2014). We therefore performed a proteomic analysis to study their functions at the proteome level. First, subcellular localization both of stable transgenic seedlings and of seedlings transiently expressing YFP- or GFP-fused proteins in Arabidopsis leaf protoplasts (from adult Col-0 plants) and tobacco leaves clearly showed that PDK1, PEL1, and HBS1 proteins located at the PM and cytoplasm (Figure 2B; Supplemental Figure S9). Intriguingly, PDK1 and PEL1 exhibited compartmentalized localization at the cytoplasm, associated with ER (Figure 2B). In addition, PEL1–YFP exhibits nuclear distribution, which was undetectable for PDK1s and HBS1 (Figure 2A; Supplemental Figure S9, A and B). The different subcellular localizations of PEL1 and HBS1 proteins suggest they might have differential functions beyond their roles in mRNA surveillance.

A tandem mass tag (TMT)-based comparative proteomics analysis was then performed using shoots and roots of 2-week-old Col-0, pdk1.1 pdk1.2 and pdk1.1 pdk1.2 pel1 seedlings, and 6,995 and 8,137 proteins were quantified in shoots (Supplemental Table S1) and roots (Supplemental Table S2). We studied shoots and roots separately because they might have different proteomes. Of the identified proteins, 71 (out of 111 changed proteins in total) and 56 (out of 113) proteins in shoots (Supplemental Tables S3 and S4) and roots (Supplemental Tables S5 and S6), respectively (Figure 5, A and B), were significantly changed in pdk1.1 pdk1.2, while rescued in pdk1.1 pdk1.2 pel1 to similar levels as in WT. These changed proteins were designated as restored commonly expressed (RCE) proteins and were speculated as being responsible for the defective growth of pdk1.1 pdk1.2.

KEGG analysis of RCE proteins revealed that pdk1.1 pdk1.2 exhibited enriched metabolic pathways, including for lipids, carbohydrates, phenylpropanoids, and amino acids, as well as enrichment of proteins involved in multiple developmental processes and environmental adaptation (Table 1; Supplemental Tables S3 and S5), which was consistent with its general growth defects. A nitrogen-regulated glutamine amidotransferase GAT 1_2.1 that represses shoot branching (Zhu and Kranz, 2012) increased in pdk1.1 pdk1.2, which is
consistent with the solitary stem phenotype of pdk1.1
pdk1.2. Furthermore, increased DUF642 family proteins
DGR1 (DUF642 L-GALL-RESPONSIVE GENE1) and DGR2
(Gao et al., 2012), and several root hair-related proteins,
including SEED AND ROOT HAIR PROTECTIVE PROTEIN,
also called ROOT HAIR-SPECIFIC 13, RHS13 (Tanaka et al.,
2017), PROLINE-RICH PROTEIN LIKE 1 (Boron et al., 2014),
GH9C1 (one GH9 gene containing a CBM49; Del Campillo
et al., 2012), DEFORMED ROOT HAIRS 9, also called
COBRA-LIKE 9, COBL9, MUTANT ROOT HAIR 4, MRH4,
SHAVEN 2, SHV2 (Ringli et al., 2005), and OXI1 (Anthony
et al., 2004; a known PDK1 substrate), may account for the
altered root development.

A large number of pathogen-induced defense-related or
systemic acquired resistance-related proteins (Thomma
et al., 1998; Ferrari, 2003; Ferrari et al., 2007; Tronchet et al.,
2010; Stotz et al., 2011; Jung et al., 2012; Breitenbach et al.,
2014; Chen et al., 2014; Weis et al., 2014) are accumulated
in pdk1.1 pdk1.2 (Table 1; Supplemental Tables S3 and S5).
Meanwhile, some abiotic stress-related proteins, especially
cold acclimation/responsive proteins (Horvath et al., 1993;
Uemura et al., 2002; Guo et al., 2013; Dyson et al., 2016)
significantly increased in pdk1.1 pdk1.2 shoots. This is con-
sistent with the previous studies showing that PDK1 posi-
tively regulates basal resistance to a blast fungus,
Magnaporthe oryzae, and a bacterial pathogen,
Xanthomonas oryzae pv. Oryzae (Xoo) in rice (Matsui et al.,
2010), and that PDK1 is required for Piriformospora indica-
duced growth promotion (Camehl et al., 2011). Together
with the previous notion that AGC kinases are major sub-
strates of PDK1 (Tan et al., 2020; Xiao and Offringa, 2020),
we conclude that these changes at the proteome level
might be indirectly regulated by PDK1 through certain
downstream pathways.

**Figure 4** Increased expression of truncated PDK1 transcripts in the pdk1.1 pdk1.2 pel1 or pdk1.1 pdk1.2 hbs1 background accounts for the rescued phenotypes. A and B, RT-qPCR analysis with primers amplifying regions across T-DNA insertions revealed that the integrity of PDK1.1 and PDK1.2 full-length CDS was disrupted by the T-DNA insertions in pdk1.1 pdk1.2, pdk1.1 pdk1.2 sop21, pdk1.1 pdk1.2 pel1, and pdk1.1 pdk1.2 hbs1. ACTIN7 gene was amplified and used as an internal control. Experiments were biologically repeated three times and data are presented as means ± SD (n = 3). Different letters represent significant differences, P < 0.05. Corrected P-values were calculated by one-way ANOVA with a Tukey’s multiple comparison test. C and D, RT-qPCR analysis with primers amplifying regions in front of T-DNA insertions revealed that N-terminal fragments of PDK1.1 and PDK1.2 transcripts (PDK1.1N and PDK1.2N) exhibited increased levels in pdk1.1 pdk1.2 sop21, pdk1.1 pdk1.2 pel1, and pdk1.1 pdk1.2 hbs1, respectively, compared to that in pdk1.1 pdk1.2. ACTIN7 gene was used as an internal control. Experiments were biologically repeated three times and data are presented as means ± SD (n = 3). Different letters represent significant differences, P < 0.05. Corrected P-values were calculated by one-way ANOVA with a Tukey’s multiple comparison test. E, Native promoter-driven expression of the PDK1 N-terminal fragment partially rescued the growth defects of pdk1.1 pdk1.2. A representative photo of 20-d-old Col-0, pdk1.1 pdk1.2, and pPDK1.1::mCherry-PDK1.1N (in pdk1.1 pdk1.2) plants grown in soil is shown. Scale bar, 2 cm.
Figure 5 Comparative proteomics showing the functions of PDK1 and PEL1 in shaping the proteomes in Arabidopsis. Heat map displays the abundance of 71 RCEs in shoots (A, proteins listed in Supplemental Table S3) and 56 RCEs in roots (B, proteins listed in Supplemental Table S5) of WT Col-0, pdk1.1 pdk1.2 and pdk1.1 pdk1.2 pel1. “pp” refers to pdk1.1 pdk1.2 double mutant and “ppp” refers to pdk1.1 pdk1.2 pel1 triple mutant. Three independent samples of WT (WT-1, -2, -3), pdk1.1 pdk1.2 (pp-1, -2, -3), and pdk1.1 pdk1.2 pel1 (ppp-1, -2, -3) were collected and analyzed. Heat maps were generated using log2-transformed TMT values. The color scale (from blue to red) indicates the protein levels. Relative level of RCE proteins was used to perform the hierarchical clustering analysis using the OECloud tools at https://cloud.oebiotech.cn.
Most RCE proteins showed increased levels (113 out of 127 proteins) in pdk1.1 pdk1.2, suggesting that PDK1 deficiency may lead to promoted protein biosynthesis. To test if PDK1 participates in the regulation of the PELOTA–HBS1 complex, we examined the protein levels of PEL1 and HBS1 in the TMT-based comparative proteomics datasets. Results showed that PEL1 and HBS1 exhibited unchanged protein abundance in pdk1.1 pdk1.2 (Supplemental Figure S10, A and B), which however can not exclude that PDK1 might regulate the PELOTA–HBS1 complex at the post-translational level.

PDK1 is an essential activator of AGC kinases, and most functions of PDK1 are mediated by AGC3 sub-family members, including D6PK/D6PKLs and PAX (Tan et al., 2020).

| Category                        | Definition                          | RCE proteins                      | pdk1.1 pdk1.2 |
|---------------------------------|-------------------------------------|-----------------------------------|---------------|
| Metabolisms                     | Lipid metabolism                    | Phospholipase A2 family protein PLA2α | De            |
|                                 |                                    | Lipoygenase LOX2                   | In            |
|                                 |                                    | Lipid transport superfamily protein AT1G23130 | In            |
|                                 |                                    | Lipid transfer protein LTP3        | In            |
| Carbohydrate metabolism         | Fumarase FUM2                       | In                                |               |
|                                 | Galactose mutarotase-like protein AT3G47800 | In                              |               |
|                                 | Alpha-galactosidase AGAL1            | In                                |               |
|                                 | Xyloglucan endotransgalactosylase/hydrolase XTH5 | In                              |               |
| Phenylpropanoid biosynthesis    | Cinnamyl alcohol dehydrogenase       | In                                |               |
|                                 | Peroxidase superfamily proteins AT1G34510 | In                             |               |
|                                 | Peroxidase superfamily proteins PER4 | In                                |               |
|                                 | Peroxidase superfamily proteins AT3G49960 | In                             |               |
| Amino acid metabolism           | Asparagine synthetase ASN3           | De                                |               |
|                                 | D-site 205 pre-rRNA nuclease AT5G41190 | In                             | De            |
| Development                     | Shoot branching                     | Nitrogen-regulated glutamine amidotransferase 1_2.1 | In            |
|                                 | Root and rosette                    | DUF 642 family proteins DGR1       | In            |
|                                 |                                    | DUF 642 family proteins DGR2       | In            |
|                                 | Root hair                           | Root hair-specific 13 SRPP         | In            |
|                                 |                                    | Proline-riched protein-like 1      | In            |
|                                 |                                    | Glycosyl hydrolase 9C1 AtGH9C1     | In            |
|                                 |                                    | Deformed root hair 9               | In            |
| Embryo                          | Embryo defective 1923 (EMB1923)      | De                                |               |
|                                 | Resurrection1 RST1                  | In                                |               |
|                                 | Yellow stripe like 3                | In                                |               |
| Chloroplast                     | Chloroplast division-related protein FAD6 | De                          |               |
| Stress                          | Pathogenesis-related defense /      | Pathogenesis-related 3            | In            |
|                                 | systemic acquired resistance        | Cinnamyl alcohol dehydrogenase     | In            |
|                                 |                                    | Yellow stripe like 3               | In            |
|                                 |                                    | Flavin-binding monoxygenase family protein FMO | In            |
|                                 |                                    | Phospholipase A2 family protein PLA2α | De            |
|                                 |                                    | Legume lectin family protein LLP1  | In            |
|                                 |                                    | Polygalacturonase inhibiting protein 2 AtPGIP2 | In            |
|                                 |                                    | Polygalacturonase inhibiting protein 1 AtPGIP1 | In            |
|                                 |                                    | CYP83A1                           | In            |
| Cold                            | Cold-responsive protein 6.6 COR6.6   | In                                |               |
|                                 | Cold-responsive protein 78 COR78     | In                                |               |
|                                 | Fumarase 2 FUM2                     | In                                |               |
|                                 | Lipid transfer protein 3             | In                                |               |
| Seed storage proteins           | 2S albumin                          | 2S seed storage protein 1 AT2S126  | In            |
|                                 |                                    | 2S seed storage protein 3 AT2S3     | In            |
|                                 |                                    | 2S seed storage protein 4 AT2S4     | In            |
|                                 |                                    | 2S seed storage protein 5 AT2S5     | In            |
|                                 | 12S globulin                        | Vicilin-like seed storage protein AT4G36700 | In            |
|                                 |                                    | Cruciferin A CRA1                  | In            |
|                                 |                                    | Cruciferin B CRB                   | In            |
|                                 |                                    | Cruciferin 2 CRU2                  | In            |
|                                 |                                    | Cruciferin 3 CRC                   | In            |
|                                 | Other nutrient reserve-related      | Lipoygenase protein LOX2           | In            |
|                                 | proteins                            | Oleosin OLEO2                      | In            |
|                                 |                                    | Seed-specific protein AT2G05580     | In            |

Proteins were functionally categorized by KEGG pathway analysis and information from previous studies. In, increased; De, decreased; SRPP, SEED AND ROOT HAIR PROTECTIVE PROTEIN.
Therefore, we checked the protein levels of the known PDK1 substrates from the AGC family in the proteomic datasets. There is an increase of D6PK protein, an essential downstream component of PDK1 (Tan et al., 2020), in pdk1.1 pdk1.2, but a relatively lower level of D6PK in pdk1.1 pdk1.2 pel1 (Supplemental Figure S11A). No dramatic changes were found for other detected AGC proteins in the proteomics data (Supplemental Figure S11, A and B). Therefore, it is very unlikely that changes of these AGC substrates might account for the rescue of pdk1.1 pdk1.2 by the pel1 mutation. In line with the notion that PDK1 activates AGC kinases via direct phosphorylation (Tan et al., 2020; Xiao and Offringa, 2020), this type of regulation does not appear to impact AGC protein stability.

**Discussion**

PDK1 is highly conserved in eukaryotes and is essential for growth and development of various organisms (Pearce et al., 2010). PDK1 deficiency results in severe growth defects or even lethality, which have impeded studies on the molecular mechanism underlying these defects, especially in mammals (Lawlor et al., 2002). By taking advantage of plant genetics and by screening for suppressors that rescue the growth defects of T-DNA insertional pdk1.1 pdk1.2 mutants, we here identify PEL1, which is a component of the PEL1–HBS1 mRNA surveillance complex and is essential for mRNA quality control during protein translation (Csorba et al., 2018). Our studies reveal that the PEL1–HBS1 complex coordinates ribosome rescue and protein biosynthesis (Figure 6A), and elucidate the mechanism for the pel1 and hbs1 mutations that suppress pdk1.1 pdk1.2 phenotype. That is, the inability of the PEL1–HBS1 complex to degrade aberrant mRNAs leads to the production of truncated but functional PDK1 proteins (Figure 6, A and B).

**Figure 6** A proposed model showing the function of PEL1–HBS1 mRNA surveillance complex and how sop21 suppresses pdk1.1 pdk1.2 phenotypes. A, The PEL1–HBS1 complex regulates 80S ribosomes (composed of a 60S large subunit and a 40S small subunit) through translational surveillance to maintain normal protein translation and plant growth. In the case of truncated PDK1 transcripts fused with partial T-DNA fragments in pdk1.1 pdk1.2, this complex could degrade these aberrant mRNAs, thus promoting the recycling of stalled 80S ribosomes. A-site, ribosomal site most frequently occupied by aminoacyl-tRNA, which functions as an acceptor for the growing protein during peptide bond formation; P-site, ribosomal site harboring decylated tRNA upon its transit out of the ribosome. B, A proposed model showing sop21 mutation rescuing the defects of pdk1.1 pdk1.2: (1) In WT, the PDK1 transcripts have the stop codon, and can be translated into 100% of PDK1 protein. (2) In the pdk1.1 pdk1.2 T-DNA mutants, aberrant transcripts with fusion to a partial T-DNA fragment will be recognized by the PEL1–HBS1 complex, and thus get degraded, exhibiting PDK1 loss-of-function mutant defects. (3) The sop21 mutation leads to the inefficient degradation of aberrant transcripts, which produce enough truncated PDK1 protein to maintain normal growth of pdk1.1 pdk1.2 plants.
surveillance mechanisms. Moreover, RST1 was recently identified as a regulator of the vacuolar protein degradation pathway (Zhao et al., 2019), implying a role of PDK1 in the endomembrane trafficking process. Interestingly, precursors of two major storage proteins, 25 albumin and 125 globulin (Shimada et al., 2003; Li et al., 2011; Table 1; Supplemental Tables S3 and S4) are accumulated in pdk1.1 pdk1.2 shoots at significantly higher levels. Likewise, a number of nutrient reserve-related proteins including lipid transfer protein LTP3 (LIPID-TRANSFER PROTEIN3; Guo et al., 2013), lipoxygenase protein LIP0XYGENASE2 (Tang et al., 2012), oleosin OLEO2 (OLEOS12; Choy et al., 2008), and seed-specific protein AT2G05580 (Umezawa et al., 2006) exhibited the same change of protein levels in pdk1.1 pdk1.2. Storage proteins are actively synthesized at rough ER as precursor forms and then are transported into protein storage vacuole (PSV) during seed maturation (Li et al., 2006). In angiosperms, seed storage proteins are deposited in PSVs of dry seeds as a source of nitrogen for growth after seed germination (Müntz, 1998; Li et al., 2006). Accumulation of seed storage proteins and nutrient reserve-related proteins in pdk1.1 pdk1.2 shoots may result in the altered vegetative growth. This indicates that PDK1 represses seed storage proteins and nutrient reserve-related proteins in the vegetative tissues or the nitrogen utilization after seed germination.

It is noteworthy that the pdk1.1 pdk1.2 phenotype is not fully rescued by pel1 mutations, or by a truncated PDK1.1N transgene. We speculate that the truncated PDK1 protein retains only partial functionality. Meanwhile, PDK1 may also regulate specific life activities through phosphorylating distinct substrates, including those well-characterized AGC kinases as well as possible other players (Zegzouti et al., 2006b; Tan et al., 2020). How these downstream pathways coordinate with each other in space and time needs further investigation.

Materials and methods

Materials and growth conditions

Arabidopsis thaliana lines used in this study were all in ecotype Columbia (Col-0) background. Seeds of Col-0 and various mutants, transgenic lines were germinated on Murashige and Skoog (Duchefa) medium after 2 d stratification at 4°C. Seedlings and plants were grown in a phytotron at 22°C with a 16-h light/8-h dark photoperiod. Root growth measurements were performed using 14-d-old seedlings grown on Murashige and Skoog medium.

Mutant lines pdk1.1-2 (pdk1.1, SALK_113251C; Tan et al., 2020), pdk1.2-4 (pdk1.2, SALK_017433; Tan et al., 2020), pdk1.2-2 (pdk2-4, SAIL_62_G04; Xiao and Offringa, 2020), pdk1.2-3 (pdk2-1, SAIL_450_B01; Xiao and Offringa, 2020), pel1 (pel1, SALK_124403C), and hbs1 (hbs1, CS857798) were obtained from ABRC (Alonso et al., 2003; Arabidopsis Biological Resource Center) and were genotyped by using corresponding LB, RP, and LP primers (Supplemental Table 7). pPDK1.1::GUS and pPDK1.2::GUS were reported previously (Tan et al., 2020). pdk1.1-14 pdk1.2-2 (=pdk1-14 pdk2-4),
*pdk1.1-13 pdk1.2-3 (=pdk1-13 pdk2-1), and pdk1.1-13 pdk1.2-2 (=pdk1-13 pdk2-4) were reported previously (Xiao and Offringa, 2020). The floral dip method (Clough and Bent, 1998) was used for plant transformation.*

**RT-qPCR analysis**

Total RNA was extracted from seedlings using TRIzol reagent (Life Technologies, 15596-018), incubated with DNase and reverse transcribed with a TAKARA Primescript™ RT Reagent Kit with gDNA Eraser (RR037A). Transcription of corresponding genes and ACTIN7 (ATSG09810, used as internal reference) was analyzed using SYBR Premix Ex Taq (TAKARA, RR420A) with a BIO-RAD CFX Connect Real-Time System. Relative expression of examined genes was first normalized to ACTIN7, and then calculated by setting the gene expression level of WT as “1”, and was presented as average ± standard deviation (sd) from three independent biological replicates.

**Promoter::β-glucuronidase staining for expression pattern analysis**

pPDK1.1::GUS and pPDK1.2::GUS transgenic lines were generated previously (Tan et al., 2020), and the pPEL1::GUS line (1.5-kb genomic sequence upstream of PEL1 ATG was used) was generated with a modified pCambia1300 binary vector (Liu et al., 2003) using primers listed in *Supplemental Table S7*. Stable transgenic lines were stained at 37°C for 1 h, in β-glucuronidase (GUS) solution [0.5-mg·ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid (X-Gluc), 0.5-mM potassium ferricyanide (K₃[Fe(CN)₆]), 0.1% (v/v) Triton X-100, 10-mM ethylenediaminetetraacetic acid (EDTA), and 0.1-M sodium phosphate (NaH₂PO₄); pH 7.0] (Tan et al., 2020). Three independent lines were analyzed in detail for different tissues and stages, and they all showed similar expression patterns. Samples were imaged by a stereomicroscope (Nikon SMZ1500).

**Yeast two-hybrid assay**

Yeast two-hybrid (Y2H) assays were performed as reported previously (Tan and Xue, 2014). The coding sequence of PEL1 was amplified by PCR with PEL1-F (BamHI)-3 and PEL1-R (BamHI) primers, and was then subcloned into the pGBKT7 vector (Clontech). Coding sequences of HBS1 [using primers HBS1-F (EcoRI) and HBS1-R (BamHI)] and HBS1 [using primers HBS1-2-F (Ndel) and HBS1-2-R (BamHI)] were amplified and subcloned into pGADT7 vectors (Clontech). Primers are listed in *Supplemental Table S7*. Bait and prey plasmids were co-transformed into the yeast strain AH109 according to the manufacturer’s introduction (Clontech). Transformants were selected on SD (-Leu/-Trp) solid medium. For auxotroph assays, four individual colonies were cultured in liquid SD (-Leu/-Trp) medium overnight, and ~10 μL of each sample at different dilutions (as indicated in the figure legends) was dropped on SD (-Leu/-Trp/-His) medium supplemented with 0.5 mg·ml⁻¹ X-α-Gal or on SD (-Leu/-Trp/-His/Ade) medium, with 1 mM 3-amino-1,2,4-triazole (3-AT), and grown at 30°C for 3 d. Colonies showing continuous growth with a blue color represented interactions.

**BiFC assay**

For BiFC assay, cDNAs encoding PDK1.1, PDK1.2, PEL1, and HBS1 were cloned into the pEYFPn vector with BP reactions. Afterward, LR reactions were conducted with the 3SS:GFP-vectors and 3SS:GFP-c vectors destination vectors (Nakamura et al., 2010), resulting in 3SS:PDK1.1-YFP, 3SS:PDK1.2-GFP, 3SS:PDK1.2-YFP, 3SS:PDK1.1-GFP, 3SS:PEL1-YFP, and 3SS:HBS1-GFP. Resultant constructs with control blank vectors were co-expressed in *Nicotiana benthamiana* leaves and yellow fluorescence was observed by a Leica SP8 confocal laser scanning microscope, using an argon laser excitation wavelength of 488 nm after infiltration for 48 h. The emission wavelengths are 520–550 nm.

**Subcellular localization and co-localization studies**

For subcellular localization studies, cDNAs encoding PDK1.1, PDK1.2, PEL1, and HBS1 were first cloned into the pENTR plasmid with BP reactions. Afterward, LR reactions were conducted with the pgWB605s destination vector, resulting in pgWB605-3SS:PDK1.1-GFP, pgWB605-3SS:PDK1.2-GFP, pgWB605-3SS:PEL1-GFP, and pgWB605-3SS:HBS1-GFP, respectively. PDK1.1-GFP, PDK1.2-GFP, PEL1-GFP, HBS1-GFP, and ER-mCherry (Nelson et al., 2007) fusion proteins were transiently expressed in *N. benthamiana* leaves (Lin et al., 2012). For mCherry fusion studies, PDK1.1 and PEL1 were cloned into the pB7m24GW2 destination vector. 3SS:GFP-HDEL was used for the ER reporter. The infiltrated leaves were harvested 2 d after infiltration and observed using an Olympus confocal microscope (Olympus, FV10i) or a LSM800 confocal laser scanning microscope (Zeiss). PDK1.1-YFP, PDK1.2-YFP, PEL1-YFP, and HBS1-YFP were cloned into the pA7 plasmid and transiently expressed in leaf protoplasts of WT Arabidopsis seedlings expressing ER-mCherry (Nelson et al., 2007), or Pip2-RFP (Yang et al., 2016). Transformed protoplasts were harvested 12 h after transformation and observed using an Olympus confocal microscope (Olympus, FV10i).

For 3SS:PDK1.1-GFP, 3SS:PDK1.2-GFP, and 3SS:PEL1-GFP transgenic plants, entry vectors were reacted with pB7FWG2.0 plasmids for GFP fusion expression. Transformation was performed with the floral dip method (Clough and Bent, 1998) with the *Agrobacterium tumefaciens* strain GV3101. More than 10 T₃ lines were selected by Basta resistance and checked by the fluorescence signal. At least three independent lines were analyzed for T₃ homozygous lines, and similar subcellular localizations were observed.

Images were captured with following excitation (Ex) and emission (Em) wavelengths (Ex/Em): GFP 488 nm/501–528 nm/YFP 490 nm/520–550 nm; mCherry/RFP 543 nm/620–630 nm; DAPI 405 nm/437–476 nm. Images are analyzed with the Fiji program (Schindelin et al., 2012).
Protein extraction and Western blot analysis
To examine the protein levels of FLAG- and GFP-tagged proteins, ~100 mg of plant tissues were frozen in liquid nitrogen, ground thoroughly, and homogenized in 100-μL protein extraction buffer [20-mM Tris–HCl, pH 7.5, 150-mM NaCl, 0.5% (v/v) Tween-20, 1-mM EDTA, 1-mM 1,4-dithiothreitol (DTT)] containing a protease inhibitor cocktail (Completé, Roche) and a protein phosphatase inhibitor tablet (PhosSTOP, Roche). After addition of sodium dodecyl sulfate loading buffer, the samples were heated at 65°C for 5 min, resolved by 10% (v/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride membranes. FLAG-tagged proteins were detected by a mouse anti-FLAG antibody (M20008, 1:2,000, Abmart). GFP-tagged proteins were detected with a mouse anti-GFP antibody (M20004, 1:2,000, Abmart) or a mouse anti-GFP HRP (horseradish peroxidase)-conjugated antibody (130-091-833, 1:2,000, MACS Molecular). Actin was detected by a mouse anti-actin antibody (M20009, 1:2,000, Abmart).

After incubation with a primary mouse antibody, the PVDF membrane was then incubated with a goat anti-mouse immunoglobulin G AP-conjugated secondary antibody (ab97020, 1:5,000, Abcam). AP activity was detected by BCIP/NBT kit (Invivogen) according to the supplier’s instructions. HRP activity was detected by the Supersignal Western Detection Reagents (Thermo Scientific).

Protein expression and purification
Coding regions of PDK1.1, PEL1, and HBS1 were amplified with corresponding primers, and subcloned into vectors pET28a (Novagen) or pGEX-4T-1 (GE Healthcare). Proteins were recombinantly expressed in *Escherichia coli* (strain BL21) by supplementing with 1-mM or 0.2-mM isopropyl-β-D-thiogalactopyranoside (induced at either 28°C for 3 h or 16°C for 16 h). Fusion proteins with His tag were purified using Ni-NTA His binding resin (Novagen) and those with GST tag were purified by glutathione sepharose (Novagen).

GST pull-down assay
GST pull-down assay for testing the interaction between GST–HBS1 and His–PEL1 was performed as reported previously (Tan and Xue, 2014; Tan et al., 2020). GST–HBS1 and His–PEL1 proteins were expressed and purified in *E. coli* (strain BL21). A 1 mL of GST (as control; this assay used relatively less volume due to a higher protein yield than GST–HBS1) and 15-mL GST–HBS1 lysates were incubated with 100-μL GST binding resin for 30 min in 1.5 mL tubes at 4°C. The supernatant was discarded. The pellet was washed with 400-μL 1×TBS (50-mM Tris–HCl, 150-mM NaCl, pH 7.6). Around 200-μL purified His–PEL1 (about 0.5 μg·μL⁻¹) was added into each tube, plus another 400-μL 1×TBS, and then incubated at 4°C for 30 min. Afterward, the samples were spun down at 3,000 g for 1 min, and then washed with 500 μL 1×TBS for three times. The resin was finally re-suspended in 30-μL elution buffer (1×TBS, 10-mM glutathione), and the sample was then added with 15-μL SDS loading dye. Twenty microliter samples were separated with 12% (v/v) SDS–PAGE, and detected by Western blot. His-tagged proteins were detected by a mouse anti-His antibody (sc-8036, 1:3,000, Santa Cruz Biotechnology). GST-tagged proteins were detected by a mouse anti-GST antibody (sc-138, 1:3,000, Santa Cruz Biotechnology).

A TMT-based comparative proteomics analysis
A TMT-based comparative proteomics analysis was performed according to Thompson’s research (Thompson et al., 2003). WT (Col-0), *pdk1.1 pdk1.2, pdk1.1 pdk1.2 pel1* seedlings were grown on 1/2 Murashige and Skoog dishes for 14 d. One gram of shoots (aerial parts) and 0.6 g roots (underground parts) of three genotypes of seedlings were set as groups 1 and 2, respectively. There are three biological replicates for each genotype.

Samples were frozen in liquid nitrogen and ground homogeneously into powder. TCA/acetone (1:9) at 5× sample volume was added to the powder and mixed by vortexing. The mixture was placed at −20°C for 4 h, and centrifuged at 6,000g for 40 min at 4°C. The supernatant was discarded. The pre-cooled acetone was added to wash three times. The pellet was air-dried. SDT buffer at 30× (30 mL for 1 mg pellet) sample volume was added to 20–30 mg powder, mixed and boiled for 5 min. The lysate was sonicated and then boiled for 15 min. After centrifuged at 14,000g for 40 min, the supernatant was filtered with 0.22-μm filters. The filtrate was quantified with the BCA Protein Assay Kit (Bio-Rad, USA). The sample was stored at −80°C.

Twenty microgram of proteins for each sample were mixed with 5× loading buffer respectively and boiled for 5 min. Proteins were separated with 12.5% (v/v) SDS–PAGE (constant current 14 mA, 90 min) and bands were visualized by Coomassie Blue R-250 staining.

Two hundred microgram of proteins for each sample were incorporated into 30-μL SDT buffer [4% SDS (w/v), 100-mM DTT, 150-mM Tris–HCl, pH 8.0]. The detergent, DTT, and other low-molecular-weight components were removed using UA buffer (8-M Urea, 150-mM Tris–HCl, pH 8.0) by repeated ultrafiltration (Microcon units, 10 kDa). Then 100-μL iodoacetamide (100-mM iodoacetamide in UA buffer) was added to block reduced cysteine residues, and the samples were incubated for 30 min in darkness. The filters were washed with 100-μL UA buffer three times and then with 100-μL 100-mM triethylammonium bicarbonate (TEAB) buffer twice. Finally, the protein suspensions were digested with 4-μg trypsin (Promega) in 40-μL TEAB buffer overnight at 37°C, and the resulting peptides were collected as a filtrate. The peptide content was estimated by UV light spectral density at 280 nm using an extinction coefficient of 1.1 of 0.1% (w/v) solution that was calculated on the basis of the frequency of tryptophan and tyrosine in vertebrate proteins.

One hundred micrograms peptide mixture of each sample was labeled using TMT reagent according to the manufacturer’s instructions (Thermo Fisher Scientific) and analyzed
on an Orbitrap Fusion Lumos (Thermo Scientific) mass spectrometer coupled with Ultimate 3000 RSLC nano system. Four microliters of each fraction was injected for nano liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. The peptide mixture (1 μg) was loaded onto the Acclaim PepMap 100 analytical column (75 μm × 15 cm, C18, 3 μm, Thermo Scientific) in buffer A [0.1% (v/v) Formic acid] and separated with a linear gradient of buffer B [80% (v/v) acetonitrile and 0.1% (v/v) Formic acid] at a flow rate of 300 nL-min⁻¹. The electrospray voltage of 2.1 kV, versus the inlet of the mass spectrometer, was used. Mass spectrometer was operated in the data-dependent mode to switch automatically between mass spectrometry (MS) and tandem mass spectrometry (MS/MS) acquisition with a cycle time of 3 s. Survey full-scan MS spectra (m/z 375–1,800) were acquired with a mass resolution of 120 K, followed by sequential high energy collisional dissociation MS/MS scans with a resolution of 50K. In all cases, one micросcan was recorded using dynamic exclusion of 40 s. For MS/MS, precursor ions were activated using 38% normalized collision energy.

MS/MS spectra were analyzed using ProteinDiscoverer Software 2.1 against the TAIR10_pep_20101214 database and decoy database with following parameters. The highest score for a given peptide mass (best match to that predicted in the database) was used to identify parent proteins. Parameters for protein searching were set as follows: trypptic digestion with at most two missed cleavages, carbamidomethylation of cysteines as fixed modification, and oxidation of methionines and protein N-terminal acetylation as variable modifications. Peptide spectral matches were validated based on q-values at a 1% false discovery rate.

Proteins were considered differentially expressed when they displayed significant changes between two groups of datasets (>1.2-fold by analysis of variance (ANOVA), p < 0.05). Adjusted p-values were calculated using the Tukey’s honestly significant difference method. Relative levels of the analyzed proteins (the values of abundance divided by 100) were used to perform the hierarchical clustering analysis using the OECloud tools at https://cloud.oebiotech.cn

The FASTA protein sequences of differentially changed proteins were blasted against the online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://geneontology.org/) to retrieve their KOs and were subsequently mapped to pathways in KEGG. The corresponding KEGG pathways were extracted.

Quantification and statistics

Lateral root numbers were counted directly. For measurements of silique length, primary root length, and leaf area, photos were analyzed with the Image J program (https://imagej.nih.gov/ij/download.html). Fluorescence intensities of reporter lines were analyzed and quantified by Fiji (https://fiji.sc/; SchindeLin et al., 2012). Data visualization and statistics were performed with GraphPad Prism®. Student’s t test was used for comparing two data sets, and one-way ANOVA with a Tukey’s test was performed for multiple comparisons. Statistical analysis for the TMT-proteomics data was performed using the OECloud tools at https://cloud.oebiotech.cn.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: PDK1.1 (AT5G04510), PDK1.2 (AT3G10540), PEL1 (AT4G27650), HBS1 (AT5G10630), D6PK (AT5G59310), D6PK1 (AT4G26610), D6PK2 (AT5G47750), D6PK3 (AT3G27580), PID (AT2G34650), OXI1/AGC2-1 (AT3G25250), GAT_1.2 (AT1G15040), SRPP (AT4G02270), PRPL1 (AT5G05500), DGR1 (AT1G80240), DGR2 (AT5G25460), GH9C1 (AT1G48930), DER9 (AT5G49270), LTP3 (AT5G59320), LOX2 (AT3G45140), and OLEO2 (AT5G0420).

Supplemental data

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

Supplemental Figure S1. Expression profiles of PDK1.1 and PDK1.2.

Supplemental Figure S2. Identification of the pel1 mutant.

Supplemental Figure S3. Expression profiles of PEL1.

Supplemental Figure S4. Loss of function of PEL1 suppresses the defective growth of pdk1.1 pdk1.2, confirmed by complementation.

Supplemental Figure S5. Sequence alignment among Arabidopsis thaliana HBS1, Homo sapiens HBS1, and Saccharomyces cerevisiae HBS1.

Supplemental Figure S6. Identification of hbs1 mutants.

Supplemental Figure S7. Phenotypes of multiple combinations of pdk1.1 pdk1.2 double mutants.

Supplemental Figure S8. Increased expression of PDK1 N-terminal fragments rescued the growth defects of pdk1.1 pdk1.2.

Supplemental Figure S9. Subcellular localization of PDK1.1-YFP, PDK1.2-YFP, PEL1-YFP, and HBS1-YFP.

Supplemental Figure S10. PDK1 doesn’t affect the amounts of PEL1 and HBS1 proteins.

Supplemental Figure S11. Abundance of AGC protein kinases in pdk1.1 pdk1.2 or pdk1.1 pdk1.2 pel1.

Supplemental Table S1. Proteins identified by analyzing shoot proteomes of Col-0, pdk1.1 pdk1.2, pdk1.1 pdk1.2 pel1 mutants by TMT-based proteomics.

Supplemental Table S2. Proteins identified by analyzing root proteomes of Col-0, pdk1.1 pdk1.2, and pdk1.1 pdk1.2 pel1 mutants by TMT-based proteomics.

Supplemental Table S3. RCEs identified in shoots by analyzing Col-0, pdk1.1 pdk1.2, and pdk1.1 pdk1.2 pel1 mutants.

Supplemental Table S4. Differentially expressed proteins in shoots identified by analyzing Col-0 and pdk1.1 pdk1.2 mutants.

Supplemental Table S5. restored CE proteins identified in roots by analyzing Col-0, pdk1.1 pdk1.2, and pdk1.1 pdk1.2 pel1 mutants.
Supplemental Table S6. Differentially expressed proteins in roots identified by analyzing Col-0 and pdk1.1 pdk1.2 mutants.

Supplemental Table S7. Primers used in this study.

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Data and materials availability

All data and materials necessary to evaluate the conclusions in the article or supplementary materials are available.

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