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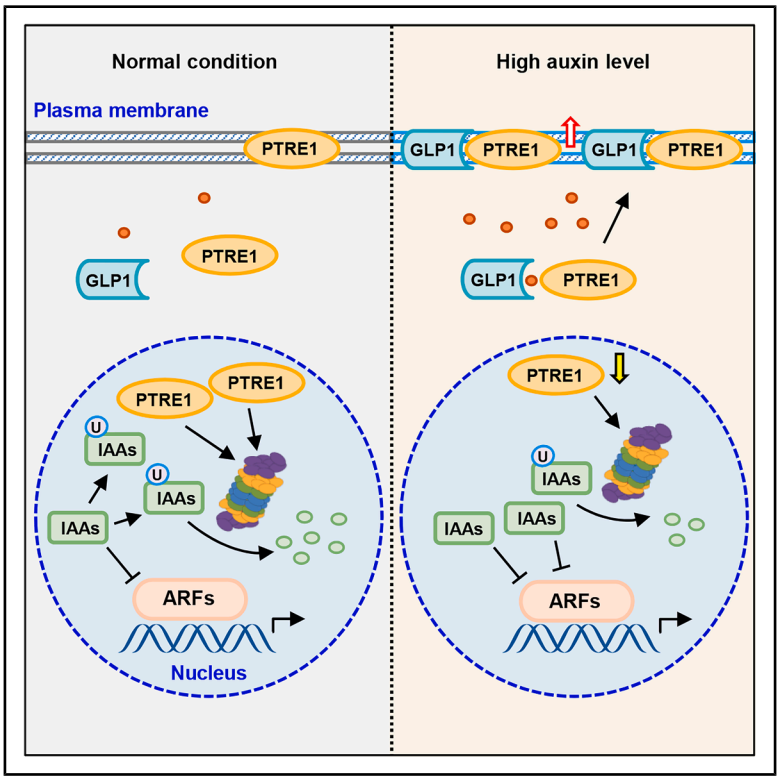
Germin-like protein 1 interacts with proteasome regulator 1 to regulate auxin signaling by controlling Aux/IAA homeostasis

Graphical abstract



Authors

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In brief

Xu et al. reveal a non-canonical mechanism that transduces the membrane-to-nucleus signal of auxin independent of the TIR1 pathway, uncovering a new layer of regulation in auxin signaling. Auxin promotes the GLP1-PTRE1 interaction, leading to PTRE1 retention at the plasma membrane and suppression of nuclear proteasome activity, thereby stabilizing Aux/IAA repressors.

Highlights

- Auxin promotes the interaction between GLP1 and proteasome regulator PTRE1
- GLP1 retains PTRE1 at the plasma membrane upon auxin stimulation
- PTRE1 relocation reduces nuclear proteasome activity and stabilizes Aux/IAAs
- The GLP1-PTRE1 axis links membrane to nuclear auxin signaling

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Germin-like protein 1 interacts with proteasome regulator 1 to regulate auxin signaling by controlling Aux/IAA homeostasis

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SUMMARY

Auxin regulates various aspects of plant growth and development by modulating the transcription of target genes through the degradation of auxin/indole-3-acetic acid (Aux/IAA) repressors via the 26S proteasome. Proteasome regulator 1 (PTRE1), a positive regulator of proteasome activity, has been implicated in auxin-mediated proteasome suppression; however, the mechanism by which auxin modulates PTRE1 function remains unclear. Here, we demonstrate that auxin promotes the interaction between germin-like protein 1 (GLP1) and PTRE1, facilitating PTRE1 retention at the plasma membrane. The relocation of PTRE1 results in reduced nuclear 26S proteasome activity, and thus the attenuated Aux/IAA degradation and altered Aux/IAA homeostasis, ultimately resulting in suppressed auxin-mediated transcriptional regulation. Our findings uncover a previously uncharacterized regulatory axis in auxin signaling that controls Aux/IAA protein stability, functioning alongside the TIR1- and TRANSMEMBRANE KINASE 1 (TMK1)-mediated pathways, and highlight the coordination of auxin signaling from the cell surface to the nucleus via auxin-induced PTRE1 relocation, which fine-tunes Aux/IAA protein homeostasis and auxin responses.

INTRODUCTION

Auxin regulates a wide range of plant developmental processes and is arguably one of the most essential signaling molecules for plant growth and adaptation.[1–5] Functioning over both long and short distances, auxin integrates multiple signaling pathways to coordinate the diverse biological processes.[6] While substantial progress has been made in elucidating how auxin is perceived and transduced at the cellular level,[7] many aspects of its complex regulatory mechanisms remain elusive.

A central feature of auxin signaling is a short yet versatile transduction cascade that translates auxin levels into the altered transcription of downstream genes. In response to auxin, hundreds of genes undergo rapid transcriptional changes.[8] The canonical nuclear pathway involves auxin-dependent interactions between the TIR1/AFB receptors[9,10] and auxin/indole-3-acetic acid (Aux/IAA) transcriptional repressors,[11–13] which leads to the ubiquitination and subsequent 26S proteasome-mediated degradation of Aux/IAA proteins,[14–18] releasing AUXIN RESPONSE FACTORs (ARFs) to activate auxin-responsive gene expression.[19–21] In addition to the well-characterized nuclear TIR1-mediated pathway, recent studies have uncovered a non-canonical auxin signaling mechanism involving TRANSMEMBRANE KINASE 1 (TMK1), which operates in parallel to the nuclear pathway. Upon auxin perception, TMK1 undergoes cleavage at the plasma membrane (PM), and its intracellular kinase domain is translocated into the nucleus to phosphorylate and stabilize the atypical Aux/IAA proteins IAA32 and IAA34, thereby repressing ARF activity.[22]

The rapid degradation of Aux/IAA proteins is tightly regulated by the ubiquitin-26S proteasome system,[23] underscoring the crucial role of proteasome activity in auxin signaling. Interestingly, although auxin promotes Aux/IAA degradation, it can also suppress 26S proteasome activity via the accumulation of PROTEASOME REGULATOR 1 (PTRE1), which is a positive regulator of 26S proteasome activity, at the PM, thereby reducing Aux/IAA degradation in the nucleus.[24] However, the molecular mechanism underlying the auxin-mediated suppression of 26S proteasome activity, particularly via PTRE1, remains largely unknown.

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Here, we demonstrated that germin-like protein 1 (GLP1), also known as auxin-binding protein (ABP1)-like protein 1 (ABL1), functions as a modulator of auxin signaling. ABL1 has been reported as a novel apoplastic ABP that forms a co-receptor complex with TMK to mediate extracellular auxin signaling.[25] We further revealed that GLP1 binds to IAA *in vitro* and interacts with PTRE1 in an auxin-promoted manner, leading to the accumulation of PTRE1 at the PM, thus suppressing 26S proteasome activity and Aux/IAAs degradation in the nucleus, thereby attenuating auxin signaling. Our findings establish GLP1-PTRE1 as a critical regulatory module of auxin signaling that functions in concert with the TIR1- and TMK1-mediated pathways to fine-tune Aux/IAA homeostasis and auxin responses in plants.

RESULTS

PTRE1 interacts with GLP1 in an auxin-promoted manner

Auxin has been reported to regulate 26S proteasome activity through PTRE1, thereby modulating the Aux/IAA-dependent transcriptional responses.[24] To investigate how auxin suppresses proteasome activity via promoting PTRE1 accumulation at the PM, we performed a yeast two-hybrid (Y2H) screen using PTRE1 as bait. Screening a cDNA library constructed from *Arabidopsis thaliana* hypocotyls yielded several candidate PTRE1-interacting proteins, including ABP1 and GLP1, a member of the GLP family.

ABP1 has been primarily associated with rapid, non-transcriptional auxin responses, and *abp1*-null mutants do not exhibit auxin-related developmental defects.[26,27] GLP1 is identical to ABL1[25] and belongs to the GLP family, whose members participate in diverse physiological and developmental processes,[28] and some members, such as GLP4, have been shown to be correlated with auxin responses.[29] These observations prompted us to focus on the potential role of GLP1 in auxin-mediated suppression of proteasome activity. Follow-up Y2H assays confirmed a direct interaction between PTRE1 and GLP1 (Figure S1A).

GLP1 encodes a 208-amino-acid polypeptide with high sequence homology to known ABPs, showing 65% similarity to peach ABP19 and 67% to peach ABP20 (Figure S1B). Sequence analysis identified three conserved motifs in GLP1, boxes A, B, and C, characteristic of the GLP protein family (Figures S1B and S1C). Notably, motifs B and C have been reported to possess auxin-binding capabilities.[29]

To investigate whether GLP1 and PTRE1 interact in plant cells, we first performed a co-immunoprecipitation (Co-IP) assay, demonstrating that GLP1 directly interacts with PTRE1 *in vivo* (Figures 1A and S2). This interaction was further validated by a bimolecular fluorescence complementation (BiFC) assay by observing *Nicotiana benthamiana* leaf epidermal cells co-expressing PTRE1-cYFP with GLP1-nYFP, or PTRE1-nYFP with GLP1-cYFP, and clear YFP fluorescence (Figure 1B), confirming a direct interaction between GLP1 and PTRE1 *in vivo*. Moreover, confocal microscopy observations of transgenic *Arabidopsis* and *N. benthamiana* lines co-expressing PTRE1-GFP and GLP1-mCherry revealed that PTRE1 and GLP1 co-localize at the PM and in the cytoplasm but are absent from the nucleus (Figures 1C and S3).

A fluorescence lifetime imaging microscopy-fluorescence resonance energy transfer (FLIM-FRET) analysis was performed to examine whether auxin affects the interaction between PTRE1 and GLP1. The average fluorescence lifetime of PTRE1-GFP alone at the PM was approximately 2.82 ± 0.06 ns, which slightly decreased to 2.75 ± 0.05 ns upon co-expression with GLP1-mCherry. Notably, in the presence of 1 μM IAA, the fluorescence lifetime was significantly reduced to 2.45 ± 0.03 ns (Figure 1D), indicating that auxin enhances the interaction between PTRE1 and GLP1 at the PM. The auxin-stimulated PTRE1-GLP1 interaction was further supported by *in vivo* coIP analysis (Figure 1E) and *in vitro* glutathione S-transferase (GST) pull-down assays (Figure 1F), with both showing the significantly enhanced interaction between GLP1 and PTRE1 in the presence of IAA and confirming that auxin directly promotes GLP1-PTRE1 association. In addition, given that GLP1 contains an auxin-binding motif, a preliminary nuclear magnetic resonance (NMR) analysis revealed a direct binding of GLP1 to IAA *in vitro* (Figure S4), which is consistent with the fact that auxin facilitates the GLP1-PTRE1 interaction and implicates a role of the GLP1-PTRE1 interaction in the regulation of auxin-mediated suppression of 26S proteasome activity.

GLP1 mediates auxin-induced suppression of 26S proteasome activity

Given its interaction with PTRE1, a known positive regulator of the 26S proteasome in auxin signaling, we examined whether GLP1 contributes to PTRE1-dependent auxin responses. A *GLP1* loss-of-function mutant, *glp1*, was identified (Figure S5A), and analysis showed that 26S proteasome activity in *glp1* under normal growth conditions was comparable to that of wild-type Col-0 (Figure 2A), which is consistent with the unaltered growth of *glp1* (Figure S6). However, unlike in Col-0, where auxin significantly reduced 26S proteasome activity, *glp1* failed to exhibit auxin-induced suppression (Figure 2A), indicating that GLP1 is required for auxin-mediated suppression of 26S proteasome activity.

To further assess the role of GLP1 in auxin-dependent regulation of Aux/IAA protein stability via the 26S proteasome, we monitored the levels of IAA-luciferase fusion proteins, as described previously.[30,31] IAA17 was selected as a representative due to its pivotal role in auxin signaling, particularly in root growth regulation.[32] In the absence of auxin, GLP1 overexpression (*GLP1ox*) lines exhibited significantly increased IAA17 protein levels compared to Col-0 (Figure 2B). Upon auxin treatment, IAA17 degradation was substantially reduced in *GLP1ox*, resulting in elevated protein levels, while *glp1* mutants displayed much lower IAA17 protein levels compared to Col-0 (Figure 2C). These findings suggested that GLP1 plays a critical role in auxin-mediated suppression of 26S proteasome activity and contributes to the regulation of Aux/IAA protein turnover.

GLP1 regulates auxin responses in plants

To explore the role of GLP1 in auxin responses, we first examined auxin-induced root growth inhibition. Compared with Col-0, *glp1* mutants showed significantly reduced sensitivity to auxin, as evidenced by the attenuated root growth inhibition (Figure 2D), indicating the negative regulatory role of GLP1 in

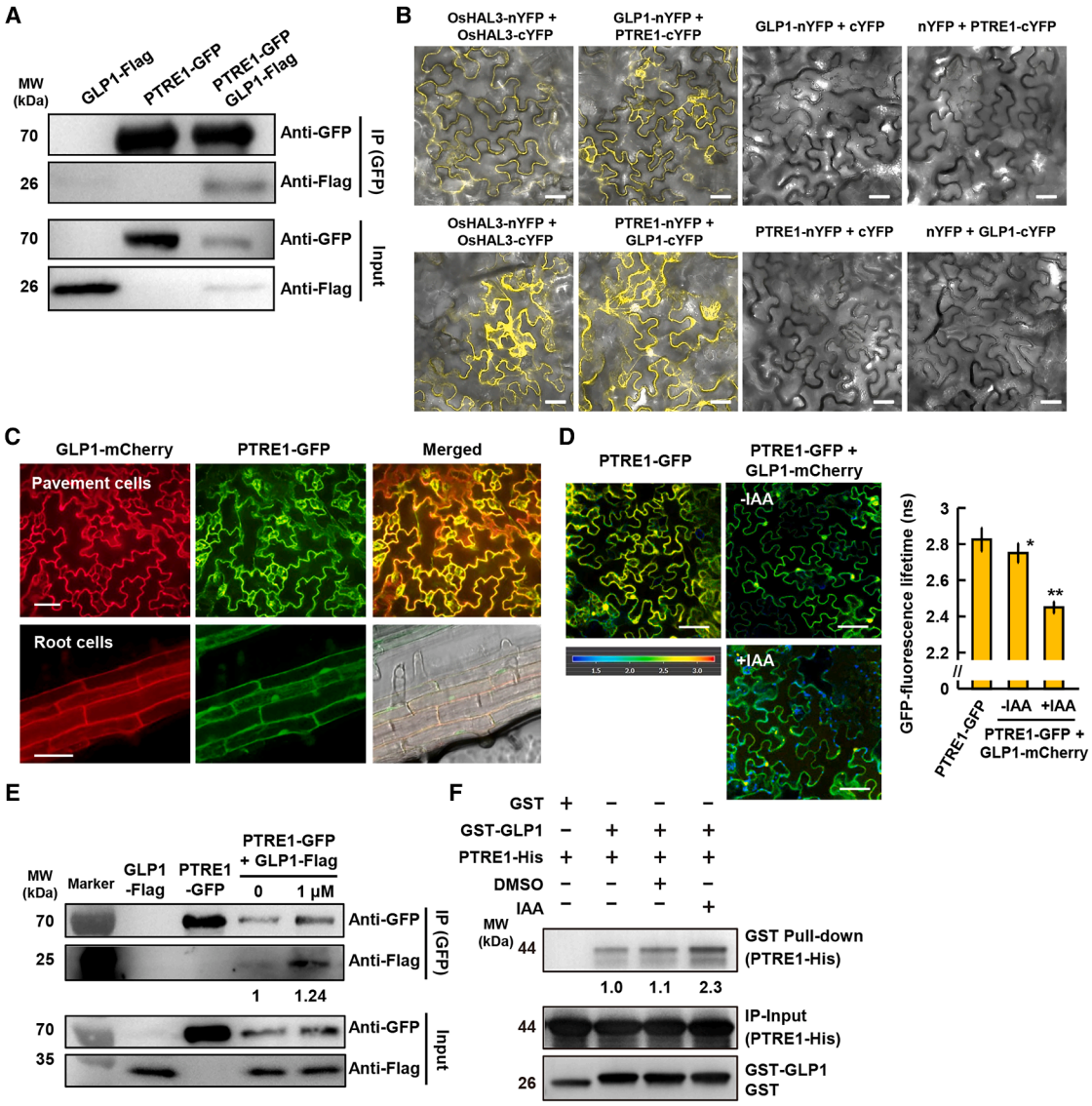

Figure 1. *Arabidopsis* GLP1 interacts with PTRE1, which is promoted by auxin

(A) Co-immunoprecipitation (coIP) analysis reveals the association of PTRE1 and GLP1 *in vivo*. Protein extracts from 7-day-old *Arabidopsis* seedlings co-expressing PTRE1-GFP and GLP1-FLAG were incubated with GFP antibody-conjugated beads and followed by an immunoblot probed with FLAG antibody. Input, total proteins. IP, immunoprecipitation.

(B) Bimolecular fluorescence complementation (BiFC) assay showed the PTRE1-GLP1 interaction *in vivo*. PTRE1 fused to the C-terminal half of yellow florescent protein (PTRE1-cYFP) and GLP1 fused to the N-terminal half of YFP (GLP1-cYFP) or, in the opposite way, were transiently co-expressed in *N. benthamiana* leaves. OsHAL3 was used as a positive control. Experiments were biologically repeated, and images with overlays of fluorescence and light views are shown. Scale bar, 40 μm.

(C) Subcellular localization of GLP1 and PTRE1. Pavement and root cells of *Arabidopsis* seedlings co-expressing PTRE1-GFP and GLP1-mCherry were observed, and representative images are shown. Scale bar, 50 μm.

(D) FLIM-FRET analysis between PTRE1-GFP and GLP1-mCherry without or with IAA (1 μM) treatment. Fluorescence lifetime of GFP was calculated from different regions of cells ($n = 10$, with 14 regions of interest in leaves expressing PTRE1-GFP, 15 regions co-expressing PTRE1-GFP/GLP1-mCherry, and 17 regions under IAA treatment). Data are presented as mean ± SEM. Statistical significance was determined by Student's t test (*$p < 0.05$ and **$p < 0.01$). Scale bar, 100 μm.

(E) coIP analysis of GLP1 and PTRE1 with or without auxin treatment *in vivo*. Proteins extracted from 14-day-old *Arabidopsis* seedlings expressing GLP1-FLAG or PTRE1-GFP or co-expressing GLP1-FLAG and PTRE1-GFP after treatment with IAA (1 μM, 1 h) were incubated with anti-GFP antibody conjugated beads and followed by an immunoblot probed with FLAG antibody. Experiments were biologically repeated. Band quantities were calculated using ImageJ, and IP protein without IAA treatment was defined as "1."

(F) Purified PTRE1-His was precipitated by GST-GLP1 using agarose beads in the absence or presence of IAA (1 μM, DMSO as control). The pellet fraction was eluted and analyzed by immunoblotting using anti-His antibody. The input of His- or GST-tagged proteins is shown. Experiments were biologically repeated. Band quantities were calculated using ImageJ, and the pull-down protein without DMSO and IAA treatment was defined as "1."

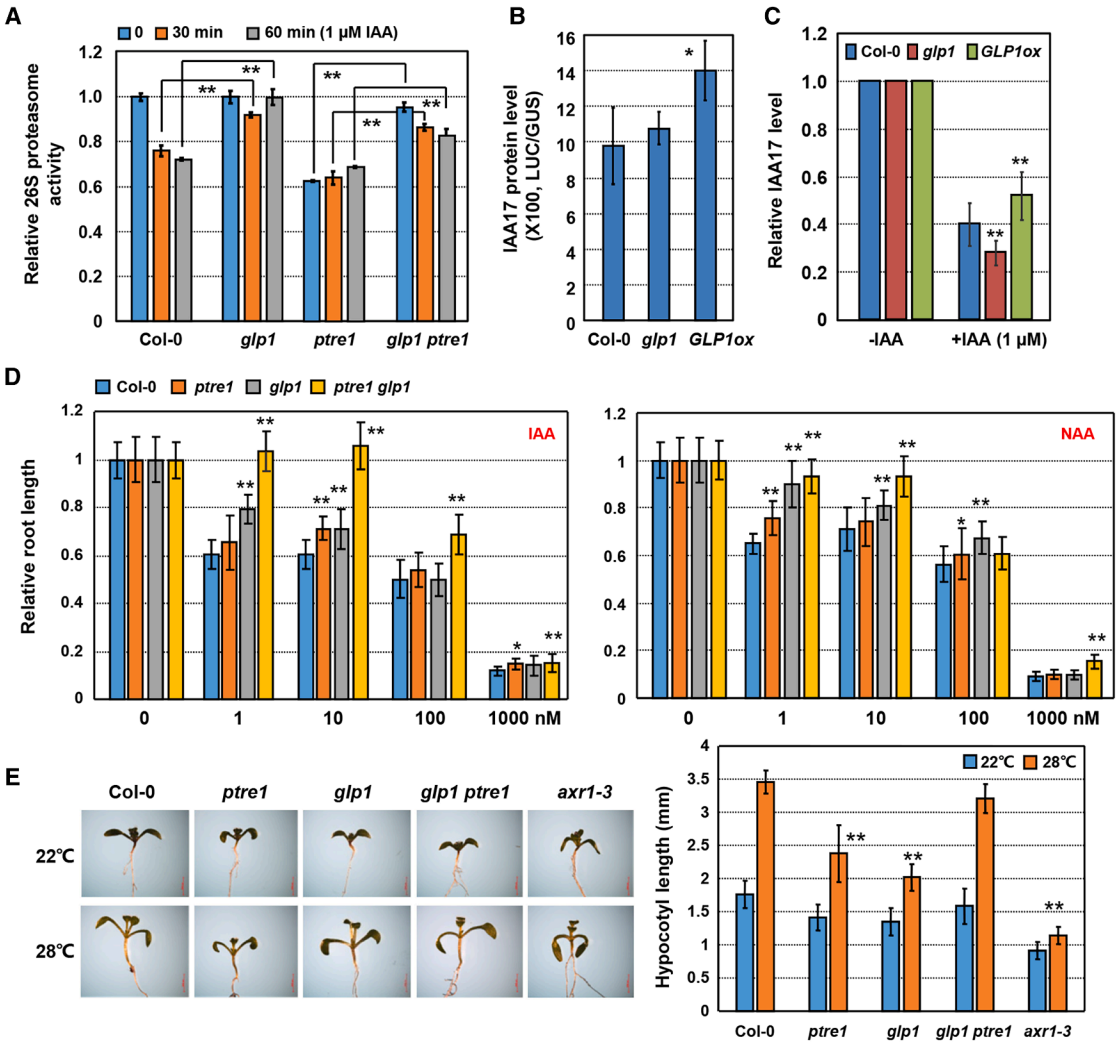

Figure 2. GLP1 is involved in auxin signaling

(A) An assay of 26S proteasome activity showed that auxin-suppressed 26S proteasome activity was inhibited in *glp1* and *glp1 ptre1*. 7-day-old seedlings of Col-0, *ptre1*, *glp1*, and *glp1 ptre1* were treated with IAA (1 μM) for 30 or 60 min, and then total proteins were extracted and used for 26S proteasome activity measurement using Suc-LLVY-AMC as the substrate. The relative 26S proteasome activity was calculated by defining the activity of Col-0 without auxin as "1.0." Experiments were biologically repeated three times, and values are the average ± SEM (n = 3). Statistical significance was determined by Student's t test (**$p < 0.01$).

(B) Increased level of IAA17 protein under GLP1 overexpression. IAA17-luciferase was transiently expressed in protoplasts of Col-0, *glp1*, or *GLP1ox* plants, and luciferase activities were examined. pUBI10:GUS was co-transformed as an internal control. Experiments were biologically repeated three times, and data are means ± SD (n = 3). Statistical significance was determined by Student's t test (*$p < 0.05$, compared to Col-0).

(C) Measurement of luciferase activity showed the increased or decreased IAA17 degradation rate in *glp1* or *GLP1ox* seedlings under IAA treatment (1 μM). IAA17-luciferase was transiently expressed in protoplasts of Col-0, *glp1*, or *GLP1ox* lines, and *pUBI10:GUS* was co-transformed as an internal control. The relative IAA17 level was calculated by defining the IAA17-luciferase amount without IAA treatment as "1.0." Experiments were biologically repeated three times, and data are means ± SD (n = 3). Statistical significance was determined by Student's t test (**$p < 0.01$).

(D) Root growth of Col-0, *ptre1*, *glp1*, and *ptre1 glp1* in the presence or absence of auxin. Root lengths of 7-day-old seedlings grown under different concentrations of IAA (left) or NAA (right) were measured. The relative length was calculated by setting that without auxin as "1.0." Experiments were biologically repeated three times, and data are presented as means ± SE ($n > 50$). Statistical significance was determined by Student's t test (*$p < 0.05$ and **$p < 0.01$, compared to Col-0 under the same conditions).

(E) Inhibited hypocotyl elongation at 28°C observed in *glp1* or *ptre1* was partially rescued in *glp1 ptre1* double mutant. 6-day-old Col-0, *ptre1*, *glp1*, and *ptre1 glp1* seedlings grown under 22°C or 28°C were observed (left), and hypocotyl length was measured using ImageJ. Data are presented as means ± SE ($n \geq 50$), and statistical analysis was performed using Student's t test (right, **$p < 0.01$, compared to Col-0 under 28°C). Auxin signaling-deficient mutant *axr1-3* was used as a control. Scale bar, 200 μm.

the auxin response. To further assess whether GLP1 affects the transcriptional responses of auxin signaling, we analyzed the expression of several *Aux/IAA* genes, which are rapidly induced by exogenous auxin.[20] Auxin-induced expression of these *Aux/IAA* genes was significantly compromised in *glp1* mutants compared to Col-0 (Figure S7), indicating that GLP1 is required for proper transcriptional regulation of auxin responses.

Under low-nutrient conditions, auxin has been shown to mildly promote hypocotyl elongation in light-grown seedlings.[33] In line with this, *glp1* mutants exhibited a diminished hypocotyl elongation response to auxin treatment (Figure S8), further supporting the notion that GLP1 is essential for fine-tuning auxin responses in plants.

GLP1 and PTRE1 antagonistically regulate auxin-mediated 26S proteasome activity and auxin responses

Auxin-induced suppression of 26S proteasome activity is mediated by PTRE1, and the loss of PTRE1 results in impaired auxin responses.[24] Given the direct interaction between GLP1 and PTRE1 and the involvement of GLP1 in auxin-mediated 26S proteasome activity regulation, we further explored their functional relationship.

To this end, we generated the *ptre1 glp1* double mutant by crossing *glp1* with *ptre1* (Figure S5B). Biochemical analysis showed that 26S proteasome activity in *ptre1 glp1* was significantly higher than in *ptre1* but slightly lower than in *glp1* (Figure 2A), indicating that PTRE1 promotes, while GLP1 suppresses, 26S proteasome activity. The partial restoration of auxin-mediated proteasome suppression in the *ptre1 glp1* double mutant further highlights and supports an antagonistic relationship between GLP1 and PTRE1 in regulating auxin-mediated 26S proteasome activity, which is critical for proper auxin signaling.

The genetic interaction of GLP1 and PTRE1 in auxin responses appeared more complex. Both *glp1* and *ptre1* mutants exhibited reduced sensitivity to auxin-induced root growth inhibition, with the *ptre1 glp1* double mutant showing an even greater insensitivity (Figure 2D), which is consistent with the fact that the induced expression of Aux/IAA genes under auxin treatment was reduced in these mutants (Figure S7). In addition, analysis of high-temperature-induced hypocotyl elongation, a process primarily dependent on auxin signaling,[34] revealed that the reduced hypocotyl elongation observed in either single mutant was partially restored in the *glp1 ptre1* double mutant (Figure 2E). The exacerbated auxin insensitivity in *ptre1 glp1* further confirms the antagonistic regulatory roles of PTRE1 and GLP1 in auxin responses, and these phenotypic inconsistencies suggest that the downstream effects of GLP1 or PTRE1 on auxin responses are context dependent. The additive effect on root growth inhibition and the rescue of high-temperature-induced hypocotyl elongation in the *ptre1 glp1* double mutant highlight the complex interaction between PTRE1 and GLP1, reflecting a possible tissue-specific or pathway-specific difference downstream of proteasome regulation.

GLP1 retains PTRE1 protein at the PM

Aux/IAA proteins primarily accumulate in the nucleus, whereas PTRE1 is localized at both the PM and nucleus, suggesting that PTRE1's subcellular localization is critical for its role in proteasome-mediated auxin signaling. Considering the opposing effects of GLP1 and PTRE1 on 26S proteasome activity and the auxin-enhanced interaction between GLP1 and PTRE1 at the PM, we hypothesized that GLP1 regulates 26S proteasome activity and Aux/IAA degradation by modulating PTRE1's subcellular distribution.

To test this hypothesis, we analyzed the PTRE1 distribution between the PM and nucleus. Consistent with previous reports,[24] auxin treatment increased PTRE1 accumulation at the PM while reducing its presence in the nucleus (Figure 3A). However, in *glp1* mutants, auxin-induced PTRE1 accumulation at the PM was significantly reduced, with a corresponding increase in the nucleus. Conversely, *GLP1ox* enhanced PTRE1 accumulation at the PM and decreased its nuclear presence (Figure 3A). Observations using PTRE1-GFP fluorescence confirmed that *GLP1ox* reduces the nuclear intensity of PTRE1-GFP (Figure 3B). Furthermore, live imaging of guard cells showed that auxin-stimulated PM distribution or decreased nuclear distribution of PTRE1 was significantly promoted under *GLP1ox* (Figure S9). In addition, western blotting analysis demonstrated auxin-induced GLP1 protein accumulation at the PM, suggesting that auxin promotes the formation of the GLP1-PTRE1 complex at PM (Figure 3C).

To determine whether the altered subcellular localization was due to the transcriptional changes, transcript levels of *GLP1* and *PTRE1* in response to auxin were examined by qPCR. Results showed that auxin treatment did not significantly alter the mRNA levels of both genes (Figure 3D), suggesting that auxin-induced redistribution of GLP1 and PTRE1 occurs post-transcriptionally and supporting the conclusion that GLP1 promotes PTRE1 accumulation at the PM through protein interactions.

GLP1 suppresses PTRE1 function to fine-tune proteasome activity and Aux/IAA protein turnover

To further elucidate the functional relationship between GLP1 and PTRE1, we examined the effect of GLP1 on auxin-regulated 26S proteasome activity. Consistent with previous findings, auxin-suppressed proteasome activity observed in PTRE1-overexpressing (*PTRE1ox*) plants was reversed by GLP1 deficiency (*PTRE1ox/glp1*) and further enhanced by *GLP1ox* (*PTRE1ox/GLP1ox*) (Figure 4A). Notably, *GLP1ox* alone also led to decreased 26S proteasome activity, while the unresponsiveness of *glp1* to auxin (Figure 2A) could not be rescued by PTRE1 overexpression (*PTRE1ox/glp1*) (Figure 4A), indicating that GLP1 is required for PTRE1-mediated suppression of proteasome activity by auxin.

We then investigated the regulation of Aux/IAA protein turnover. Quantification of the IAA17-luciferase fusion protein revealed that IAA17 levels were elevated in *PTRE1ox/GLP1ox* plants and reduced in *PTRE1ox/glp1* plants compared to *PTRE1ox* alone (Figure 4B), confirming the negative regulatory role of GLP1 on PTRE1 function and that GLP1 suppresses 26S proteasome activity and auxin response through PTRE1. Further genetic analysis by observing the responses to auxin-induced root growth inhibition revealed that in *PTRE1ox* plants, *GLP1ox* (*PTRE1ox/GLP1ox*) suppressed, whereas GLP1 deficiency (*PTRE1ox/glp1*) enhanced, auxin sensitivity, as indicated

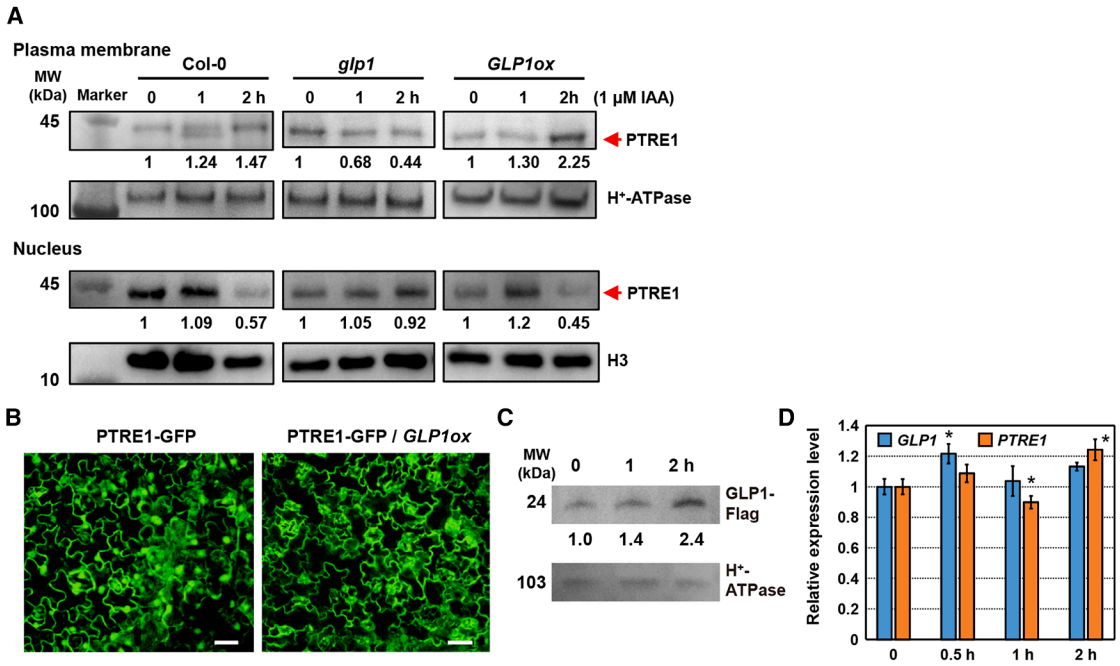

Figure 3. Auxin promotes PTRE1 accumulation at plasma membrane

(A) PTRE1 proteins in plasma membrane (PM) and nucleus fractions were examined by western blot analysis. Proteins were extracted from *GLP1ox* or *glp1* seedlings treated without or with auxin (1 μM, 1 or 2 h). PM or nucleus fractions were verified by antibodies against H^+-ATPase or H3, respectively. Experiments were biologically repeated. Band quantities were calculated using ImageJ. The relative amount of PTRE1 protein was normalized first to H3 or H^+-ATPase and then to the control sample (without IAA treatment was defined as "1.0").

(B) GLP1 overexpression reduces the fluorescence intensity of PTRE1-GFP in nucleus. GFP fluorescence of leaf epidermal cells from 7-day-old Col-0 or *GLP1ox* seedlings expressing PTRE1-GFP was observed. Experiments were biologically repeated two times, and representative images are shown. Scale bar, 20 μm (left) or 50 μm (right).

(C) Examination of GLP1 protein at PM by western blot analysis. Proteins were extracted from seedlings expressing GLP1-FLAG treated without or with auxin (1 μM, 1 or 2 h). PM fractions were verified by using antibody against H^+-ATPase. Band quantities were calculated using ImageJ, and GLP1 proteins of seedlings without IAA treatment were defined as "1.0."

(D) Relative transcript levels of *GLP1* and *PTRE1* in 7-day-old Col-0 seedlings treated with 1 μM IAA for 0.5, 1, and 2 h were measured by qPCR analysis normalized with ACTIN. The relative expression was calculated by defining the expression of examined genes at time 0 as "1." Experiments were biologically repeated three times, and values are means ± SD ($n = 3$). Statistical significance was determined by Student's t test (*$p < 0.05$, compared to time 0).

by altered root growth responses (Figure 4C). These findings demonstrate that GLP1 fine-tunes the auxin response by negatively regulating PTRE1 function, thereby modulating 26S proteasome activity and Aux/IAA protein turnover.

DISCUSSION

Based on our findings, we propose a working model in which auxin regulates Aux/IAA protein homeostasis not only through the canonical nuclear TIR1/AFB receptor pathway[35–37] but also via a GLP1-PTRE1 regulatory module that modulates 26S proteasome activity. Auxin promotes GLP1 accumulation at the PM and strengthens its interaction with PTRE1, thereby retaining PTRE1 at the PM. This spatial sequestration limits PTRE1's nuclear function, resulting in reduced 26S proteasome activity in the nucleus and attenuated degradation of Aux/IAA proteins, ultimately fine-tuning auxin signaling at the transcriptional level (Figure 5).

It is noticed that the *glp1 ptre1* mutant exhibits similar activity of the 26S proteasome to *glp1* rather than *ptre1* (Figure 2A), which suggests that GLP1 may suppress 26S proteasome activity through additional mechanisms beyond PTRE1 retention. Future studies should explore whether GLP1 interacts with other proteasome components or is involved in post-translational modifications that regulate 26S proteasome function. In addition, our findings suggest that excessive degradation of Aux/IAA proteins does not necessarily lead to enhanced auxin signaling. Instead, a precise balance of Aux/IAA protein degradation is required for proper transcriptional regulation of downstream genes. In the *glp1* mutant, PTRE1 is more nucleus localized, leading to increased IAA17 degradation (Figure 2C). However, this does not translate into enhanced auxin signaling; rather, it results in auxin insensitivity, likely due to transcriptional dysregulation.

The 26S proteasome is a central regulator of multiple hormonal signaling pathways and cell responses to various environmental stimuli. As previously discussed,[24] the 26S proteasome is not exclusive to auxin signaling but also regulates key components of other hormone signaling pathways, including DELLA proteins, the key suppressors of gibberellin signaling[38]; JAZ proteins, repressors of jasmonic acid signaling[39]; ABI transcription

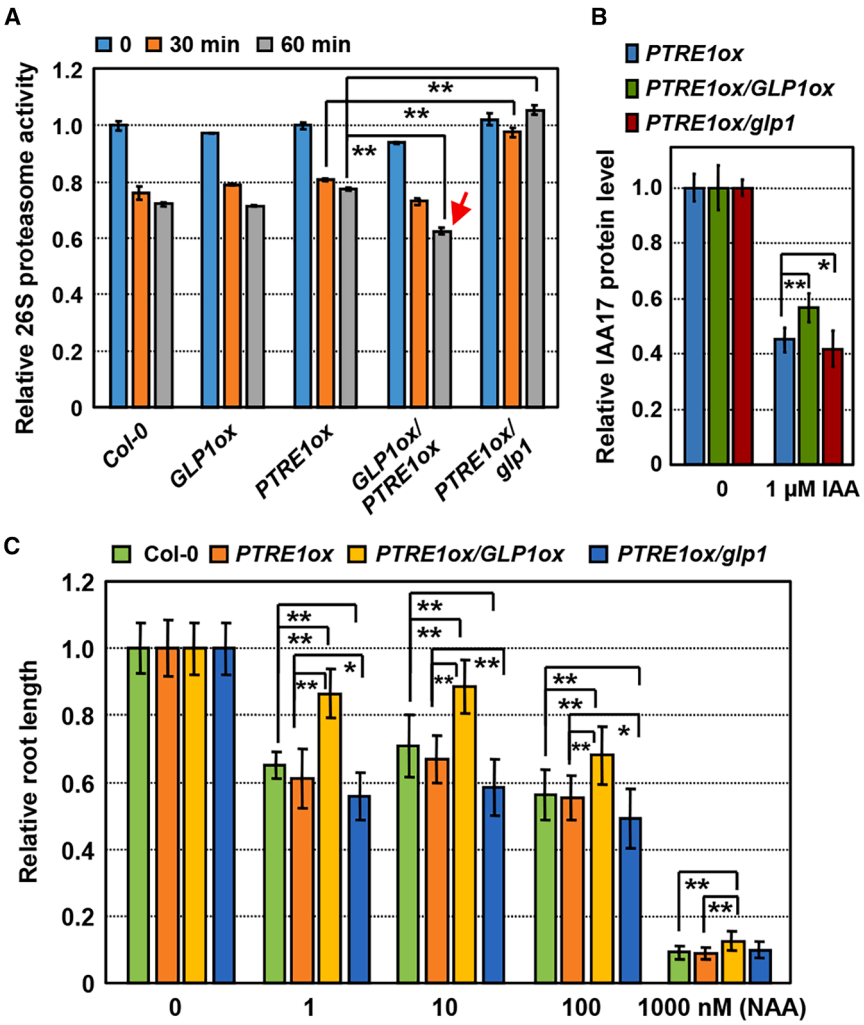

Figure 4. GLP1-PTRE1 interaction regulates auxin signaling

(A) An assay of 26S proteasome activity revealed that GLP1 deficiency (*glp1*) reversed the auxin-suppressed 26S proteasome activity in Col-0 plants overexpressing PTRE1 (*PTRE1ox*), whereas GLP1 overexpression (*GLP1ox*) further enhanced the suppression (arrow). 7-day-old seedlings of Col-0, *GLP1ox*, *PTRE1ox*, *GLP1ox/PTRE1ox*, and *PTRE1ox/glp1* were treated with IAA (1 μM) for 30 or 60 min, and then total proteins were extracted and used for measurement of 26S proteasome activity using Suc-LLVY-AMC as the substrate. The relative activity was calculated by defining the 26S proteasome activity of Col-0 without auxin as "1.0." Experiments were biologically repeated three times, and values are means ± SEM (n = 3). Statistical significance was determined by Student's t test (**$p < 0.01$).

(B) Measurement of luciferase activity showed the increased or decreased IAA17 degradation rate in *PTRE1ox/GLP1ox* or *PTRE1ox/glp1* under IAA treatment (1 μM). IAA17-luciferase was transiently expressed in protoplasts of *PTRE1ox*, *PTRE1ox/GLP1ox*, or *PTRE1ox/glp1*, and *pUBI10:GUS* was co-transformed as an internal control. The relative IAA17 level was calculated by defining the IAA17-luciferase amount without IAA treatment as "1.0." Experiments were biologically repeated three times, and values are the average ± SD (n = 3). Statistical significance was determined by Student's t test (*$p < 0.05$ and **$p < 0.01$).

(C) *Arabidopsis* seedlings overexpressing PTRE1 and GLP1 (*PTRE1ox/GLP1ox*) are less sensitive to auxin-suppressed root growth. Root length was measured, and the relative length was calculated by setting that absence of auxin as "1.0." Experiments were biologically repeated three times, and values are means ± SE ($n > 50$). Statistical significance was determined by Student's t test (*$p < 0.05$ and **$p < 0.01$, compared to Col-0 under the same conditions).

factors of ABA signaling,[40] and proteins of brassinosteroid and strigolactone signaling. Given the importance of PTRE1 in regulating 26S proteasome activity, regulation of PTRE1 by GLP1 in response to auxin may also impact the response to other hormones, which positions PTRE1 a key hub in hormonal signaling pathways, where distinct regulatory mechanisms may fine-tune PTRE1's activity in response to different developmental or environmental cues, ensuring precise regulation of plant development and stress responses.

Interestingly, based on yeast screening results and sequence homology between GLP1 and ABP1, we found that auxin also enhances the interaction between ABP1 and PTRE1 (Figure S10A). While *abp1* mutants do not exhibit canonical defects in auxin signaling or development, recent studies indicate that ABP1 functions in rapid auxin responses.[26] However, genetic analysis showed that *abp1 ptre1* mutants did not restore suppressed hypocotyl elongation at high temperatures (Figure S10B), suggesting that the ABP1-PTRE1 interaction may not be involved in canonical auxin signaling. Further studies will help to determine whether the ABP1-PTRE1 interaction regulates alternative auxin-regulated processes. Recent studies indicated that auxin promotes ABP1 clustering in the apoplast, forming co-receptors with TMK1 to mediate extracellular auxin perception,[25,26] raising the possibility that auxin might act as a molecular glue, facilitating GLP1 clustering at the PM to retain PTRE1 or inhibit PTRE1's endocytosis.

In sum, this study proposes a potential auxin signaling pathway mediated by the GLP1-PTRE1 axis, distinct from the TIR1-mediated pathway, to regulate Aux/IAA homeostasis. Whether TMK1-based signaling, also initiated at the cell surface, interacts with this pathway warrants further exploration. Given the critical roles of PTRE1 and the 26S proteasome in plant growth[41] and stress responses,[42] multiple regulatory mechanisms may converge on PTRE1 and the 26S proteasome to achieve precise control of plant development and environmental adaptation.

Limitations of the study

While time-course experiments were performed to monitor the protein behavior, most analyses of auxin signaling responses

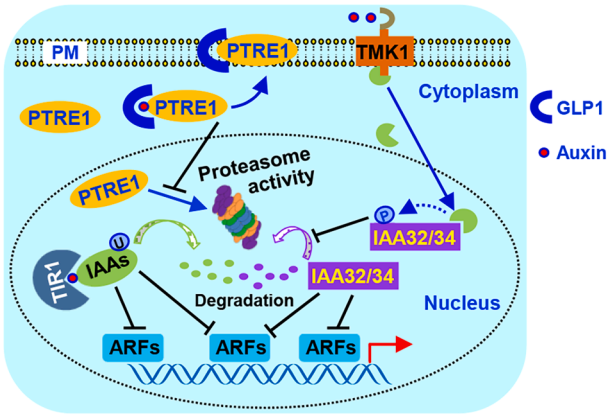

Figure 5. Proposed working model of GLP1-PTRE1-auxin signaling

A functional model illustrating how GLP1-PTRE1 interaction suppresses 26S proteasome activity and functions coordinately with TIR1 to regulate Aux/IAA homoeostasis and auxin signaling. Under increased auxin, nuclear receptor TIR1/AFB binds auxin to stimulate the degradation of Aux/IAA proteins via the 26S proteasome. GLP1 interacts with PTRE1 and promotes the plasma membrane accumulation of PTRE1, leading to suppressed activity of 26S proteasome and, hence, reduced degradation of Aux/IAA proteins in nucleus. The TMK1 intracellular kinase domain is cleaved from the plasma membrane and translocated to the nucleus to phosphorylate and stabilize atypical IAA proteins. These pathways coordinate and regulate the homeostasis of Aux/IAA proteins, resulting in fine-controlled transcription of downstream genes and auxin responses.

were conducted under steady-state conditions. This may not fully reflect the dynamic nature of signal transduction. In future studies, we will employ inducible expression systems to enable more precise temporal control and dynamic tracking of auxin-responsive signaling events. These additions will help refine our understanding of the temporal regulation and mechanistic nuances of auxin signaling.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hongwei Xue (hwxue@sjtu.edu.cn).

Materials availability

All plant materials and plasmids generated in this paper will be shared by the lead contact upon request. This study did not generate new unique reagents.

Data and code availability

- The paper did not generate a new dataset.
- This study does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

F.X. and B.G. performed the experiments. Y.Y. and T.X. provided the materials. F.X. performed the acquisition and analysis of data and drafted the article. H.X. and T.X. were responsible for the conception and design and for writing the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-H3	Agrisera	Cat# AS10710; RRID:AB_10750790
Anti-H^+-ATPase	Agrisera	Cat# AS07260; RRID:AB_1031584
Anti-PTRE1	Our previous work, Yang et al.[24]	N/A
Anti-His	Santa Cruz	Cat# sc-8036
Anti-Flag	Abmart	Cat# M20008
Anti-GFP	Abmart	Cat# M20004
Anti-GST	Santa Cruz	Cat# sc-138
Anti-Rabbit-HRP	Santa Cruz	Cat# sc-2004
Anti-Rabbit-AP	Santa Cruz	Cat# sc-2007
Anti-Mouse-AP	Abcam	Cat# ab97020
Bacterial and virus strains		
AH109 yeast	WEIDI	Cat# YC1010
Agrobacterium strain GV3101	WEIDI	Cat# AC1001
Escherichia coli strain DH5a	WEIDI	Cat# DL1001
Escherichia coli strain BL21	TIANGEN	Cat# CB105
Biological samples		
Arabidopsis thaliana – Columbia-0 (Col-0)	N/A	N/A
A. thaliana: ptre1	ABRC	SALK_034353
A. thaliana: glp1	NASC	GABI_820G11
A. thaliana: abp1TD1	Gao et al.[27]	N/A
A. thaliana: abp1c1	Gao et al.[27]	N/A
A. thaliana: ptre1glp1	This study	N/A
A. thaliana: ptre1abp1TD1	This study	N/A
A. thaliana: abp1TD1glp1	This study	N/A
A. thaliana: ptre1abp1TD1glp1	This study	N/A
A. thaliana: axr1-3	ABRC	CS3075
A. thaliana: tir1-1	Our previous work, Yang et al.[24]	N/A
A. thaliana: p35S:GLP1-mCherry (*glp1*)	This study	N/A
A. thaliana: pGLP1:GLP1-mCherry (*glp1*)	This study	N/A
A. thaliana: p35S:PTRE1-GFP (*ptre1*)	Our previous work, Yang et al.[24]	N/A
A. thaliana: p35S:PTRE1-GFP p35S:GLP1-mCherry (*ptre1*)	This study	N/A
A. thaliana: p35S:PTRE1-GFP (*glp1*)	This study	N/A
A. thaliana: pGLP1:GLP1-Flag (*glp1*)	This study	N/A
A. thaliana: p35S:GLP1-Flag (*glp1*)	This study	N/A
A. thaliana: p35S:PTRE1-GFP p35S:GLP1-Flag (*ptre1*)	This study	N/A
Chemicals, peptides, and recombinant proteins		
MS medium	Duchefa Biochemie	Cat# M0222
Indole-3-Acetic Acid (IAA)	Sigma-Aldrich	Cat# I3750
1-Naphthaleneacetic Acid (NAA)	Sigma-Aldrich	Cat# N0640
SD-Trp-Leu medium	Clontech	Cat# 630417
X-α-Gal	GOLD Biotech	Cat# 107021-38-5

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
X-Gluc	GOLD Biotech	Cat# G128C1
TRIzol	Invitrogen	Cat# 15596018
Suc-LLVY-AMC	Millpore	Cat# 539142
Luciferase Assay System	Promega	Cat# E1500
4-Methylumbelliferyl β-D-glucuronide (MUG)	Sigma-Aldrich	Cat# M9130
Anti-Flag Affinity gel	Sangon	Cat# D111139
Ni-NTA Agarose	Qiagen	Cat# 30210
glutathione agarose resin	Sigma-Aldrich	Cat# G4510
Critical commercial assays		
PrimeScript RT Reagent Kit	Takara	Cat# RR047A
SYBR Green qPCR kit	Toyobo	Cat# QPK-201
BCA Protein Assay Kit	ThermoFisher	Cat# 23225
CellLytic PN extraction kit	Sigma	Cat# CELLYTPN1
ProteoPrep® Membrane extraction kit	Sigma	Cat# PROTMEM
Gateway™ LR Clonase™ Enzyme Mix	Thermo Scientific	Cat# 11791019
pENTR Directional TOPO Cloning kit	Invitrogen	Cat# K2400-20
Oligonucleotides		
Primers (see Table S1)	This study	N/A
Recombinant DNA		
GLP1-AD	This study	N/A
GLP1-BD	This study	N/A
PTRE1-AD	This study	N/A
PTRE1-BD	This study	N/A
GLP1-nYFP	This study	N/A
GLP1-cYFP	This study	N/A
PTRE1-nYFP	This study	N/A
PTRE1-cYFP	This study	N/A
35S:GLP1-Flag	This study	N/A
35S:GLP1-mCherry	This study	N/A
pGLP1:GLP1-mCherry	This study	N/A
pGLP1:GLP1-Flag	This study	N/A
PTRE1-His	This study	N/A
GLP1-GST	This study	N/A
ABP1-His	This study	N/A
Software and algorithms		
Clustal X	European Bioinformatics Institute (EBI)	https://www.ebi.ac.uk/Tools/msa/clustalw/
ImageJ	National Institutes of Health (NIH)	https://imagej.nih.gov/ij/
LAS X	Leica Microsystems	https://www.leica-microsystems.com/products/microscope-software/
cellSens Dimension	Olympus Corporation	https://www.olympus-lifescience.com/en/software/cellsens/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Plant materials, growth condition and treatments

Columbia ecotype of *A. thaliana* (Col-0) was used as wild type. transfer DNA insertional mutants *ptre1* (SALK_034353), *glp1* (GABI_820G11) were obtained from the *Arabidopsis* Biological Resource Center[43] and the European Arabidopsis Stock Center (NASC, Nottingham, UK). Homozygous *glp1* line was identified by RT-PCR (primers GLP1-F/GLP1-R). Double mutant *ptre1 glp1*

was generated by genetic crossing and verified by genotyping. *glp1* mutant was genotyped (primers O8409, GLP1-RP and GLP1-LP). Mutants *abp1TD1* and *abp1c1* were obtained from Gao et al.[27] and used for genetic cross with *glp1* and *ptre1*. Primers are listed in Table S1.

All seeds were germinated on 1/2 Murashige and Skoog medium after two days at 4°C. Seedlings and plants were grown in a growth chamber (22°C, 16-h light/8-h dark photoperiod). Root/hypocotyl length of 7-day-old seedlings grown on media supplemented with NAA or IAA, or hypocotyl length of seedlings grown at 22°C or 28°C for 6 days, was measured by ImageJ. For auxin treatment, 7-day-old seedlings grown vertically on 1/2 MS medium were transferred to liquid 1/2 MS medium containing IAA or NAA (1 μM) for different times before extracting proteins or RNAs.

METHOD DETAILS

Yeast two-hybrid assay

Coding sequences of GLP1 and PTRE1 were fused in-frame with GAL4 DNA binding domain of bait vector pGBKT7 (Clontech) and transcriptional activating domain of prey vector pGADT7 (Clontech). Paired vectors were transformed into AH109 yeast strains on the synthetic defined (SD)-Trp-Leu medium (Clontech, Cat: 630417) and positive clones were randomly selected and transferred to SD-Leu-Trp-His and SD-Leu-Trp-His-Ade medium supplemented with 0.5 mg/mL X-α-Gal for further growth.

Bimolecular fluorescence complementation assay

GLP1 or PTRE1 was fused to C or N terminus of yellow florescent protein (YFP) and transformed to *Agrobacterium* strain GV3101. *Nicotiana benthamiana* plants were infiltrated and leaves were observed after 2 days using an Olympus confocal microscope (Olympus, FV10i). Images were captured with the following excitation (Ex) and emission (Em) wavelengths (Ex/Em): YFP, 508 nm/524 nm.

Constructs and *A. thaliana* transformation

For transient expression in *N. benthamiana* leaves, GLP1 cDNA was amplified by PCR (primers GLP1mCherryF/GLP1mCherryR) using total cDNA of *Arabidopsis* seedlings as template and cloned into pCAMBIA1300 to generate *p35S:GLP1-mCherry* construct. For *Arabidopsis* transformation, GLP1 cDNA was amplified (primers GLP1-FlagF/GLP1-FlagR) and cloned into pGWB411 to generate *p35S:GLP1-Flag* construct. Additionally, we replaced the 35S promoter with the native GLP1 promoter, which was amplified from the genomic sequence, to construct *pGLP1:GLP1-mCherry* and *pGLP1:GLP1-Flag* vectors. Transformation of Col-0, *glp1* or *ptre1* plants was performed by floral dipping procedure. Primers are listed in Table S1.

For protein expression in *Escherichia coli*, full-length coding region of GLP1, PTRE1, ABP1 genes ending with a TAA stop codon were amplified and subcloned into pGEX-4T-1, pET-51b(+) and pET-32a(+) vectors respectively, as specified in Table S1.

Subcellular localization and co-localization studies

PTRE1-GFP and GLP1-mCherry fusion proteins were transiently expressed in *N. benthamiana* leaves and observed after infiltration for 2 days using a Leica SP8X microscope. Stably transformed *A. thaliana* plants co-expressing PTRE1-GFP and GLP1-mCherry were used to observe the co-localization of PTRE1 and GLP1. Images were captured with the following excitation (Ex) and emission (Em) wavelengths (Ex/Em): GFP, 488 nm/501–528 nm; mCherry, 543 nm/620–630 nm.

Live cell imaging and FRET-FLIM analysis

Live-stoma guard cell imaging was captured with a Leica SP5 microscope and recorded. FLIM-FRET experiments[44,45] were performed using a Leica SP8X microscope equipped with fluorescence lifetime system (PicoQuant). PTRE1-GFP alone was used as donor and its fluorescence lifetime was measured as negative control. GLP1-mCherry was the fluorescence receptor and all measurements were taken from whole field images expressing fluorescence protein with similar levels. Fluorescent lifetime was calculated from at least 10 independent measurements.

Real-time quantitative PCR (qPCR) analysis

Total RNAs were isolated from Col-0, *glp1*, *ptre1* or *glp1 ptre1* seedlings using TRIzolR reagent (Invitrogen, Cat: 15596018). Complementary DNA was synthesized using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Cat: RR047A). Transcription of *Aux/IAAs*, *GLP1*, *PTRE1*, and *ACTIN2* (as the internal control) genes was analyzed using the SYBR Green qPCR kit (Toyobo, Cat: QPK-201) with a RotorGene 3,000 system (Corbett). qPCR data for each sample were normalized to the level of respective *ACTIN2* expression. Primers are listed in Table S1.

26S proteasome activity assay

Activity assay of 26S proteasome *in vivo* was performed according to previous description.[46] In detail, 7-day-old *Arabidopsis* seedlings were treated with 1 μM IAA for different times. Collected samples were ground in protein-extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM ATP, 1% Triton X-100 and 20% glycerol) and cell debris were removed by centrifugation at 10,000 g (4°C). Protein concentration was determined by BCA assay (bicinchoninic acid, Pierce, BCA Protein Assay Kit, ThermoFisher, Cat:

23225). To measure the 26S proteasome activity, 100 μg protein of crude extracts was diluted with buffer (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 2 mM ATP and 2 mM DTT) to a final volume of 1 mL (assayed in triplicate), and incubated with proteasome fluorogenic peptide substrate Suc-LLVY-AMC (Millpore, Cat: 539142) for 2 h at 37°C.

All reactions were carried out in a 96-well black plate and read by a Varioskan Flash micro-plate reader (Thermo Scientific) at excitation 380 nm and emission 460 nm.

Isolation of nucleus and plasma membrane fractions and western blotting analysis

Seven-day-old Col-0, *GLP1ox* and *glp1* seedlings were treated with 1 μM IAA for different times, collected and ground in liquid nitrogen. Proteins were extracted as previously described.[24] The nucleus and plasma membrane fractions were extracted using Sigma CellLytic PN extraction kit (Sigma, Cat: CELLYTPN1) and ProteoPrep Membrane extraction kit (Sigma, Cat: PROTMEM). Western blot analysis was performed using corresponding primary antibodies (anti-H3, cat-AS10710, 1:1,000, Agrisera; anti-H^+-ATPase, cat-AS07260, 1:1,000, Agrisera; anti-PTRE1, 1:1,000, Abmart). Band intensity was quantified by ImageJ (version 1.80).

Protein expression and purification from *E. coli*

E. coli BL21 (DE3) strains containing constructs expressing different proteins were first cultured in LB liquid medium with appropriate antibiotics until the OD_{600} reached 0.6, followed by induction with isopropyl β-D-1-thiogalactopyranoside (IPTG). GST, ABP1-His and GST-GLP1 proteins were induced with 0.3 mM IPTG at 37°C for 1.5 h, and PTRE1-His proteins were induced overnight with 0.1 mM IPTG at 18°C.

BL21 cells were harvested by centrifugation and lysed by sonication. GST-tagged proteins were affinity-purified from lysates using glutathione agarose resin, followed by elution with a buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM reduced glutathione, and 1 mM DTT. His-tagged proteins were purified using Ni-NTA Agarose (Qiagen), followed by elution with a buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 10 mM Imidazole. Purified proteins were diluted with buffer containing 15% (v/v) glycerol and stored at −80°C.

Co-immunoprecipitation (Co-IP) and pull-down analysis

Co-IP assay was performed using *N. benthamiana* and *Arabidopsis*. Plants expressing GLP1-Flag or PTRE1-GFP and plants co-expressing GLP1-Flag and PTRE1-GFP were ground to powder in liquid nitrogen and solubilized with lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1× protease cocktail inhibitors). Protein extracts were incubated with Anti-Flag magnetic beads or GFP-Trap magnetic beads at 4°C for 1 h. Immunoprecipitated proteins were washed three times with washing buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA), and resuspended with loading buffer for western bolting analysis. Antibodies against Flag (cat-M20008, 1:5,000, Abmart) or GFP (cat-M20004, 1:5,000, Abmart) were used for IP or western blot analysis.

For GST pull-down analysis, recombinant GST, GST-GLP1 and PTRE1-His proteins were used. GST, GST-GLP1 lysates were incubated with 100 μL GST resin for 30 min, then centrifuged at 3,000 rpm for 1 min. The pellet was washed three times. PTRE1-His was added into each tube and incubated at 4°C for 3 h. Beads were washed 5 times with wash buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA), boiled in 2× SDS sample buffer, and analyzed by immunoblotting using anti-His antibody.

For semi-*in vivo* pull-down assay, protein extracts were prepared from 7-day-old *Arabidopsis* seedlings expressing PTRE1-GFP using extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, and protease inhibitors). Extracts were incubated with His-tagged ABP1 (ABP1-His) conjugated to Ni-NTA agarose beads at 4°C for 1 h with gentle rotation in the presence of NAA or IAA. Blank His-beads without ABP1-His were used as a negative control. After incubation, beads were washed three times with washing buffer (same as extraction buffer but with reduced detergent concentration) to remove nonspecific binding. Bound proteins were eluted using elution buffer containing imidazole and subjected to SDS-PAGE, followed by immunoblot analysis using anti-GFP (for detecting PTRE1-GFP) and anti-His (for detecting ABP1-His) antibodies. The input control for PTRE1-GFP was included to verify protein loading.

Protoplast transfection and fluorometric LUC and GUS assays

Protoplast transfection and activity assay of IAA17-LUC fusion proteins in Col-0, *glp1*, *GLP1ox*, *PTRE1ox*, *PTRE1ox/GLP1ox*, *PTRE1ox/glp1* were carried out according to previous description.[47,48] Briefly, leaves from plants (3–4 weeks) were harvested to isolate protoplasts and 300 μL of protoplasts suspension (about 2×10^5 protoplasts) was transfected with constructs p35S::IAA17-Luc and pUBI10:GUS[24] (as internal control for normalization). Transfected protoplasts were incubated at 22°C for 12 h (low light) in the presence of IAA. After collection and lysis, LUC activity was measured using 100 μL LUC assay reagent (Promega) and 20 μL lysate with luminometer (Varioskan Flash micro-plate reader, Thermo Scientific). GUS activity was measured using 10 μL lysate and 100 μL fluorescence MUG substrate mix for 1 h at 37°C with a fluorometer (365 nm/455 nm, Varioskan Flash micro-plate reader, Thermo Scientific). Experiments were biologically repeated three times and at least three measurements were performed for each sample.

Sequence alignment analysis

GLPs were obtained via a BLAST search at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Conserved protein sequences were obtained through a BLAST search by aligning multiple-sequences using Clustal X program (downloaded from http://bips.u-strasbg.fr/fr/Documentation/ClustalX/). Conserved motifs were referred to previous description.[29]

Nuclear magnetic resonance (NMR) analysis

GLP1-Flag protein was extracted from *Arabidopsis* seedlings expressing GLP1-Flag (*GLP1ox*) and purified by using Anti-Flag Affinity gel (Sangon). Total proteins were extracted as below: seedlings were ground in buffer [50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 0.1% Nonidet P-40, 20% glycerol and cocktail] and cell debris were removed by spinning at 13,000g (4°C). The supernatant samples were incubated with Anti-Flag Affinity gel for 30 min. The gel was washed with elution buffer (0.1 M glycine HCl, pH 2.0–2.8). Antibody against Flag (cat-M20008, 1:5,000, Abmart) was used for western blot analysis.

A total of 100 mM GLP1-Flag protein and 10 mM IAA were used for a nuclear magnetic resonance (NMR) assay according to Yin et al.[29] One-dimensional spectra were recorded with nt = 32, then water-LOGSY spectra with nt = 512. After addition of protein, the corresponding parameters were recorded. All spectra were recorded at 293 K with a Varian Inova 600 MHz NMR spectrometer. Binding with protein was determined when the spectrum of auxin was oppositely altered.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Student's t-test (*, $p < 0.05$; **, $p < 0.01$), with significant differences indicated by different asterisks. Data are presented as mean ± SD or SEM, and detailed statistical information is provided in the figure legends.