Differential Regulation of B2-type CDK Accumulation in *Arabidopsis* Roots

Yoko Okushima¹, Kohei Shimizu¹, Takashi Ishida², Keiko Sugimoto² and Masaaki Umeda¹,³*

*corresponding author

¹Nara Institute of Science and Technology, Graduate School of Biological Sciences, Takayama 8916-5, Ikoma, Nara 630-0192, Japan; ²RIKEN Center for Sustainable Resource Science, Tsurumi, Yokohama, Kanagawa 230-0045, Japan; ³JST, CREST, Ikoma, Nara 630-0192, Japan

*Corresponding Author

Masaaki Umeda

Phone: +81-743-72-5592  FAX: +81-743-72-5599

Email: mumeda@bs.naist.jp
Abstract

Root growth depends on cell proliferation in the apices, which determines the root meristem size. The expression of B2-type cyclin-dependent kinase (CDKB2) is known to be restricted to dividing cells in the meristematic region, and therefore, the mechanisms controlling CDKB2 accumulation may be associated with those determining the meristem size. We investigated how CDKB2 expression is controlled in distinct zones of Arabidopsis roots. We found that CDKB2;1 expression was induced by a member of the PLETHORA (PLT) family of transcription factors, which are known to mediate auxin signaling and maintain the undifferentiated state of meristematic cells. When the root meristem was treated with an auxin antagonist, the CDKB2;1 level was reduced not only by transcriptional suppression but also by proteasome-mediated protein degradation. This indicates that auxin promotes CDKB2 accumulation at both mRNA and protein levels in the meristem. In the elongation and differentiation zones, on the other hand, neither the ubiquitin-proteasome system nor the PLT-mediated transcriptional regulation are associated with CDKB2;1 accumulation. Both CDKB2;1 and HIGH PLOIDY2 (HPY2), a SUMO E3 ligase, were ectopically accumulated in the stele when treated with exogenous auxin, suggesting the possibility that CDKB2;1 accumulation is dependent on HPY2-mediated sumoylation, which is usually maintained by a higher auxin level in the meristem. Our results demonstrate that the CDKB2 level is tightly controlled by multiple pathways to maintain the mitotic activity in developing roots.
Key words

Arabidopsis thaliana, auxin, cyclin-dependent kinase (CDK), root, meristem

Key message

The accumulation of the mitotic B2-type CDK is tightly controlled by multiple pathways in Arabidopsis roots.

Abbreviations

GFP, Green fluorescent protein; GUS, β-glucuronidase; IAA, indole-3-acetic acid; NAA, 1-naphthylacetic acid; RT-PCR, Reverse transcription-polymerase chain reaction
Introduction

Plant development depends on a fine balance between cell proliferation and differentiation. Root growth is determined by the cell proliferation rate and the subsequent cell elongation (Beemster and Baskin 1998). A growing root consists of four distinct zones: the meristematic zone, which resides at the apex and consists of stem cells and actively-dividing stem cell-descendants; the elongation and differentiation zones containing cells that expand and differentiate rather than divide; and the transition zone, suggested to be located between the meristematic zone and the elongation zone and involved in defining meristem size (Verbelen et al. 2006). The timing of the onset of cell expansion and differentiation is an important determinant of root growth (Ubeda-Tomas et al. 2009).

Although the molecular mechanisms that determine root zonation are not fully understood, recent studies have identified ways to distinguish the meristematic zone from the elongation zone. Auxin distribution plays a crucial role in root patterning and growth (Baluska et al. 2010). An auxin concentration gradient is established with a maximum at the stem cell niche (Sabatini et al. 1999; Blilou et al. 2005). Members of the PLETHORA (PLT) AP2 family of transcription factors follow a distribution gradient that partially overlaps with the auxin gradient. They determine the stem cell niche and maintain stem cell activity (Aida et al. 2004; Galinha et al. 2007). The interdependent PLT and PIN-FORMED (PIN) genes, the latter of which encode auxin efflux carriers, constitute a feedback loop that sustains the auxin gradient in roots (Blilou et al. 2005; Benjamins and Scheres 2008). Another phytohormone, cytokinin, acts antagonistically to auxin in the transition zone and plays a key role in defining the meristem size (Dello Ioio et al. 2007; Dello Ioio et al. 2008). Recent studies have shown that gibberellins and brassinosteroids affect the positioning of the transition zone, suggesting that these hormones are also responsible for specifying the size of the meristematic zone (Fu and Harberd 2003; Gonzalez-Garcia et al. 2011; Hacham et al. 2011).

In the elongation zone, a process named endoreduplication occurs. Cells go through the endocycle—a different type of cell cycle that skips the M phase (Inze and De Veylder 2006). Therefore, the meristem size is defined by the transition from the mitotic cell cycle (in the meristematic zone) to the endocycle (in the elongation and differentiation zones). Cell cycle progression is controlled by the
protein kinase activities of cyclin-dependent kinase (CDK) complexes that comprise the catalytic CDK and the regulatory cyclin subunits. In plants, the A- and B-type CDKs (CDKA and CDKB) are known to be crucial for cell cycle regulation. CDKA is a functional homolog of yeast Cdc2/Cdc28p and is expressed constitutively throughout the cell cycle. By contrast, CDKB is a plant-specific CDK and is further classified into two subtypes—CDKB1 and CDKB2. The expression patterns are stage-specific; CDKB1 and CDKB2 are expressed from the late S phase to the M phase and from the G2 to the M phase, respectively (Menges et al. 2005; Segers et al. 1996). Based on these oscillations of expression and kinase activity during the cell cycle, CDKA is likely to participate in both the G1/S and G2/M transitions, whereas CDKB functions in the late S-to-M phase (Inze and De Veylder 2006; Van Leene et al. 2010).

In agreement with the mitotic functions of the CDKBs, their expression is restricted to dividing cells in the meristematic region (Martinez et al. 1992; Adachi et al. 2009; Boudolf et al. 2004; Ishida et al. 2009; Ishida et al. 2010; Adachi et al. 2006; Andersen et al. 2008; Adachi et al. 2011). CDKA, on the other hand, is more broadly expressed in roots and is also found in the pericycle, which is competent for cell division (Hemerly et al. 1993). We previously reported that the accumulation of CDKB2 is regulated both at the transcriptional level and by proteasome-mediated protein degradation (Adachi et al. 2006), suggesting that the CDKB2 level is tightly controlled to sustain root meristem function. Here, we report that CDKB2 accumulation is controlled by multiple ways, such as transcriptional regulation, protein degradation and probably sumoylation, which are associated with auxin signaling. Our results provide insight into molecular mechanisms determining the mitotic activity in roots.

Materials and Methods

Plant material and growth conditions

All wild type and transgenic Arabidopsis thaliana lines were in the Columbia background. Seeds were surface-sterilized in 5% sodium hypochlorite with 0.15% Tween-20 and plated on Murashige–Skoog (MS) medium containing 1% sucrose solidified with 0.5% gelan gum. Plants were grown at 23°C under continuous light conditions. For chemical treatments, Arabidopsis seedlings were transferred to MS media containing indicated chemicals. Arabidopsis MM2d cells (Menges and Murray 2002) were
cultured in 1× MS medium supplemented with 200 mg L⁻¹ potassium dihydrogen phosphate, 100 mg L⁻¹ myo-inositol, 1 mg L⁻¹ thiamine hydrochloride, 3% sucrose, and 0.2 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (pH 5.8) at 27°C on a shaking incubator. For chemical treatment, five-days old seedlings were transferred to vertically oriented plates containing 10 µM PEO-IAA, 1 µM NAA, 100 µM MG132 or control solvent (ethanol).

Microscopy

For GUS staining, the root tissue was incubated in 50 mM sodium phosphate pH 7.2, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, and 0.5 mg mL⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid at 37°C for 8 min (pCDKA;1:CDKA;1-GUS), 10 min (pCDKB1;1:CDKB1;1-GUS) or 24 hr [pCDKB2;1:CDKB2;1(nt)-GUS]. Samples were cleared in a mixture of 8 g chloral hydrate, 1 ml of glycerol, and 2 ml of water and observed under a light microscope equipped with Nomarski optics. To detect GFP fluorescence, root samples were counterstained with 10 µg mL⁻¹ propidium iodide, and fluorescence images were obtained using a confocal laser-scanning microscope (Zeiss LSM710).

Results

To first examine the protein-level expression of CDKB2;1, one of the two CDKB2 in Arabidopsis, we used the pCDKB2;1:CDKB2;1(nt)-β-glucuronidase (GUS) reporter line, which expresses the first exon of CDKB2;1 fused to GUS under its own promoter. We previously reported that it can monitor the CDKB2;1 expression at the protein level, and exhibits similar expression pattern to that of the full-length CDKB2;1-GUS reporter line in the root meristem (Adachi et al. 2006; Adachi et al. 2011). As shown in Fig. 1C, the GUS signals showed a patchy pattern in the root meristem. Treatment with the proteasome inhibitor MG132 increased the accumulation of CDKB2;1(nt)-GUS (Fig. 1F), indicating that CDKB2 is degraded via the ubiquitin–proteasome pathway in plant cells (Adachi et al., 2006). To test the possibility that the other CDKs are also controlled by proteasome-dependent pathway, we examined CDK accumulation in Arabidopsis roots by using GUS reporter lines; pCDKA;1:CDKA;1-GUS and pCDKB1;1:CDKB1;1-GUS express full-length CDKA;1 and CDKB1;1, respectively, fused to
GUS, under their own promoters (Adachi et al. 2009). The GUS staining in the primary root meristem was uniform in the mock-treated roots for both CDKA;1 and CDKB1;1, and was not affected by MG132 treatment (Figs. 1A, B, D, E). These results suggest that the accumulation of CDKB2 but not of CDKA or CDKB1 is regulated by proteasome-mediated protein degradation. Expression of 

\[ pCDKA;1:CDKA;1-GUS \]

in the root cap implies the potential competency for cell division.

Auxin is a phytohormone that promotes cell proliferation in the meristem (Ishida et al. 2010). Therefore, we next examined the possibility that auxin signaling controls the expression level of CDKs. We used the auxin antagonist PEO-IAA, a compound blocking the TIR1-mediated auxin signaling (Hayashi et al. 2008). In \[ pCDKA;1:CDKA;1-GUS \] and \[ pCDKB1;1:CDKB1;1-GUS \] lines, the GUS staining in the primary root meristem was strong and uniform in both the mock- and PEO-IAA-treated roots (Figs. 2A, B, E, F). By contrast, the GUS signals in \[ pCDKB2;1:CDKB2;1(\text{nt})-GUS \] line were significantly repressed by treatment with 10 \( \mu \)M PEO-IAA for 6 h (Figs. 2C, G). Application of MG132 together with PEO-IAA abolished the PEO-IAA-induced reduction in CDKB2;1 level (Fig. 3), suggesting that CDKB2;1 is more actively degraded via the ubiquitin-proteasome pathway when auxin signaling is blocked. Indeed, the expression of \[ pCDKB2;1:GUS \], a transcriptional GUS-fusion that monitors \[ CDKB2;1 \] promoter activity (Adachi et al. 2006), was slightly decreased by PEO-IAA treatment but to a much lesser extent than that of \[ pCDKB2;1:CDKB2;1(\text{nt})-GUS \] (Fig. 2D, H). These results demonstrate that, although auxin is required for the full activation of \[ CDKB2;1 \] transcription, it is also involved in the moderate suppression of protein degradation to maintain a sufficient amount of CDKB2;1 for mitosis. We also examined the effect of PEO-IAA treatment on the expression of mitotic cyclins, \[ CYCB1;1 \] and \[ CYCB1;2 \]. As shown in Supplemental Figure 1, PEO-IAA treatment slightly suppressed the accumulation of both CYCB1;1-GUS and CYCB1;2-GUS, and this effect was abolished by simultaneous application of MG132. These observations suggest that auxin has a role in suppressing excess protein degradation of at least several mitotic regulators.

We then focused on the CDKB2;1 expression in the elongation and differentiation zones of roots. As shown in Fig. 4A, the expression of \[ pCDKB2;1:GUS \] was detected not only in the meristem but also in the transition and elongation zones, especially in the stele. On the other hand, the GUS expression in
pCDKB2;1:CDKB2;1(nt)-GUS roots was restricted to the meristematic region (Fig. 4E). Surprisingly, the expression pattern of pCDKB2;1:CDKB2;1(nt)-GUS was not affected by MG132 except slightly increased accumulation in the meristematic region (Fig. 4A, B and E, F). This indicates that post-transcriptional mechanisms other than proteasome-mediated protein degradation prevent CDKB2;1 from accumulating in the stele.

Treatment with 1 µM NAA for 12 h slightly increased the expression of pCDKB2;1:GUS in the stele (Fig. 4A and C). This only modest induction of CDKB2;1 transcription is likely to be a result of enhanced cell division activity in the pericycle by auxin treatment. Another possibility is that exogenous auxin promotes ectopic expression of PLT transcription factors, which then up-regulate the CDKB2;1 expression. To test this possibility, we introduced pCDKB2;1:GUS and pCDKB2;1:CDKB2;1(nt)-GUS into PLT2-GR line (Galinha et al., 2007), and first examined the effects of the ectopic expression of PLT2 on CDKB2;1 expression. While induction of PLT2-GR by dexamethasone (DEX) resulted in a substantial increase in the size of the proximal meristem (Galinha et al., 2007), the area of CDKB2 expression expanded to the differentiation zone (stele) for both pCDKB2;1:GUS and pCDKB2;1:CDKB2;1(nt)-GUS (Fig. 5). This indicates that CDKB2;1 expression is induced at the transcriptional level downstream of PLTs. We then observed PLT2 expression in the presence of exogenous auxin. Although the expression of pPLT2:PLT2-GFP slightly expanded in the meristematic region by 1 µM NAA treatment, it remained very faint in the stele (Fig. 6). This result indicates that a higher CDKB2;1 accumulation in the stele by auxin application is not caused by ectopic expression of PLTs.

As described above, in pCDKB2;1:CDKB2;1(nt)-GUS roots, no GUS expression was observed in the elongation and differentiation zone in the absence of exogenous auxin (Fig. 4E), but a strong expression was noted in the stele when 1 µM NAA was applied (Fig. 4G). Although this is also likely to be a consequence of enhancement of cell divisions by the auxin treatment, the drastic accumulation of pCDKB2;1:CDKB2;1(nt)-GUS (Figs. 4E and G) cannot be explained solely by a slight increase in the CDKB2;1 promoter activity (Figs. 4A and 4C); because the protein-level accumulation was much prominent compared to the mRNA level. Thus, the above-mentioned post-transcriptional mechanisms
that suppress CDKB2;1 accumulation in the stele may become disordered by NAA treatment, resulting in higher CDKB2;1(nt)-GUS accumulation. We previously demonstrated that a SUMO E3 ligase, HIGH PLOIDY2 (HPY2), promotes the mitotic cell cycle in the root meristem. In the hpy2-1 mutant, the protein accumulation of mitotic regulators, such as CDKB1;1, CDKB2;1, CYCB1;1 and CYCB1;2, were significantly suppressed (Breuer et al. 2010; Ishida et al. 2009). Especially, the expression of CDKB2;1-GUS reporter in the root meristem was almost completely abolished in the hpy2-1 mutant (Ishida et al. 2009). This suggests the possibility that HPY2 is involved in auxin-dependent CDKB2;1 accumulation in the stele. Therefore, we examined pHYP2:HPY2:GFP expression in seedlings treated with auxin. As reported previously, HPY2:GFP expression was restricted to the meristematic region in the absence of auxin (Ishida et al. 2009). Upon treatment with 1 µM NAA for 12 h, however, the HPY2:GFP protein was highly accumulated in the stele (most probably in the pericycle) (Fig. 7). This expression pattern is similar to that of pCDKB2;1:CDKB2;1(nt)-GUS in the presence of 1 µM NAA (Fig. 4G), suggesting a possibility that HPY2 is associated with the ectopic CDKB2;1 accumulation in the stele. Interestingly, in the presence of auxin, a region devoid of GUS expression was observed above the meristematic zone of both pCDKB2;1:CDKB2;1(nt)-GUS and pHYP2:HPY2:GFP lines (Fig. 4G, Fig. 7B). However, no such “blank” region was visible in pCDKB2;1:GUS (Fig. 4C), and MG132 treatment did not result in CDKB2;1 accumulation in the “blank” region (Fig. 4H). These results suggest that HPY2 is not expressed in the “blank” region even in the presence of exogenous auxin, thus CDKB2;1 is not accumulated in this particular zone. Taken together, HPY2-mediated smoylation may promote CDKB2;1 accumulation in the elongation and differentiation zones of auxin-treated roots, except in the “blank” region where unknown mechanism(s) absolutely inhibits the HYP2 expression.

Discussion

In this study, we revealed that levels of CDKB2, unlike those of CDKA and CDKB1, are regulated not only at the transcriptional level but also at the protein level. In the root meristematic zone, CDKB2;1 accumulation is controlled by proteasome-mediated protein degradation, which is moderately suppressed by auxin signaling. While the machinery for CDKB2;1 degradation remains unknown, the
PEST-like motif in the N-terminal region may be responsible for the degradation (Adachi et al. 2006). Further studies will identify the amino acid residues required for the regulation of CDKB2;1 stability. Also, studies with transgenic plants expressing a non-degradable form of CDKB2;1 will reveal how the proteasome-mediated control of the CDKB2 level is involved in the adjustment of root growth to various environmental conditions.

Auxin distribution plays a crucial role in the patterning of root tissues. Members of the PLT family of transcription factors are involved in auxin signaling (Blilou et al. 2005; Benjamins and Scheres 2008). The expression patterns of PLT genes in the root exhibit a gradient with the highest expression in the apex. This expression pattern partially overlaps with the auxin gradient (Galinha et al. 2007). Indeed, auxin is known to induce PLT expression (Aida et al. 2004). In the present study, we show that ectopic overexpression of PLT2 induced the CDKB2;1 expression, suggesting that the transcription of CDKB2;1 is regulated by PLT transcription factors downstream of auxin signaling. This indicates that, in the meristematic region, both the mRNA and protein levels of CDKB2;1 are positively controlled by auxin signaling; this mechanisms may be important to maintain the mitotic activity in stem cell descendants.

HPY2 encodes a SUMO E3 ligase, which is expressed in the root meristem under the control of PLT transcription factors (Ishida et al., 2009). We previously found that CDKB2;1 accumulation is drastically reduced in the hpy2 knockout mutant, suggesting that HPY2-mediated sumoylation is required for stabilization of CDKB2;1 protein in the meristematic region (Ishida et al. 2009). We found that the expression patterns of HPY2 and CDKB2;1 proteins well overlapped in roots treated with or without auxin; especially, both proteins were accumulated in the stele when treated with exogenous auxin. However, this ectopic expression pattern was not observed for PLT2, suggesting that exogenous auxin upregulates HPY2 expression in the stele via a PLT-independent pathway and stabilizes CDKB2;1. Therefore, under normal growth conditions, lower auxin level in the elongation and differentiation zones suppresses HPY2 expression, thus may inhibit CDKB2;1 accumulation, although CDKB2;1 is transcribed in the stele.

In this study, we identified a region devoid of CDKB2;1 accumulation above the meristematic zone, probably encompassing the transition zone and the most proximal region of the elongation zone.
Although $CDKB2;1$ is transcribed in this “blank” region, the protein does not accumulate even in the presence of exogenous auxin. The same was true for HPY2 protein, indicating that $Arabidopsis$ deploys a mechanism to abolish HPY2-mediated $CDKB2;1$ stabilization in this particular area. Since PLT expression was clearly observed in the presence of exogenous auxin, PLTs are not involved in this regulation. It remains unknown how HPY2-mediated $CDKB2;1$ stabilization is suppressed, but such a distinct regulatory system would be essential to accomplish transition from the mitotic cell cycle to the endocycle and to precisely determine the meristem size.

Auxin is traditionally referred to as a growth factor or a mitogen, and has long been known to activate cell division (John 2007). Previous studies reported that the stability of E2F transcription factors, which govern G1/S transition, S-phase progression, etc., is regulated by auxin (Magyar et al. 2005; del Pozo et al. 2002). However, little is known about how core CDK/cyclin complexes are controlled by auxin signaling. In this study, we demonstrated that auxin controls cell division by modulating the accumulation of mitotic regulators. Especially for CDKB2, its accumulation is controlled at multiple levels, such as transcriptional induction downstream of PLTs, proteasomal degradation, and perhaps HPY2-mediated sumoylation, in auxin-dependent and -independent pathways. Further studies will reveal how these mechanisms contribute to root growth under various nutritional and environmental conditions.

**Acknowledgements**

We thank Dr. Ben Scheres for the $35S:PLT2-GR$ seeds, Dr. Yoshikatsu Matsubayashi for the $pPLT2:PLT2-GFP$ seeds, and Dr. Kenichiro Hayashi for PEO-IAA. This research was supported by Grants-in-Aid for Scientific Research on Priority Areas (No. 18056006 to M. U. and No. 20053013 to Y.O.) and on Innovative Areas (No. 22119009 to M. U.), and by Grant-in-Aid for Young Scientists (Nos. 20770033 and 22770040 to Y.O.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
References


Breuer C, Ishida T, Sugimoto K (2010) Developmental control of endocycles and cell growth in...


Zone. Plant Signal Behav 1 (6):296-304
Figure legends

**Figure 1.** Proteasome-mediated protein degradation regulates accumulation of CDKB2, but not of CDKA or CDKB1.

Five-day-old seedlings carrying pCDKA;1:CDKA;1-GUS (A, D), pCDKB1;1:CDKB1;1-GUS (B, E), or pCDKB2;1:CDKB2;1(nt)-GUS (C, F) were treated with ethanol (A-C) or 100 µM MG132 (D-F) for 6 h. Root tips were stained for GUS. Bar = 50 µm.

**Figure 2.** Expression of CDKs in response to PEO-IAA treatment.

Shown are GUS-stained root meristems of seedlings harboring pCDKA;1:CDKA;1-GUS (A, E), pCDKB1;1:CDKB1;1-GUS (B, F), pCDKB2;1:CDKB2;1(nt)-GUS (C, G) or pCDKB2;1:GUS (D, H). Five-day-old seedlings were treated with 10 µM PEO-IAA (+PEO-IAA: E-H) or with ethanol only (mock: A-D) for 6 h. Bar = 100 µm.

**Figure 3.** Expression of CDKB2;1 in the root meristem.

Five-day-old seedlings carrying pCDKB2;1:CDKB2;1(nt)-GUS were treated with ethanol (B), 100 µM MG132 (C), 10 µM PEO-IAA (D), or 10 µM PEO-IAA and 100 µM MG132 (E) for 6 h. A is a 0 h control sample. Root tips were stained for GUS. Bar = 50 µm.

**Figure 4.** Observation of CDKB2;1 expression in a wide range of root tips.

Five-day-old seedlings carrying pCDKB2;1:GUS (A-D) or pCDKB2;1:CDKB2;1(nt)-GUS (E-H) were treated with ethanol (A, E), 100 µM MG132 (B, F), 1 µM NAA (C, G), or 1 µM NAA and 100 µM MG132 (D, H) for 12 h. Roots were stained for GUS. Bar = 100 µm.

**Figure 5.** Expression of CDKB2 in PLT2-overexpressing roots.

Six-day-old seedlings carrying 35S:PLT2-GR and pCDKB2;1:GUS (A) or pCDKB2;1:CDKB2;1(nt)-GUS (B) were treated with 1 µM DEX for the indicated times and
stained for GUS. Bar = 100 μm.

**Figure 6.** Expression of PLT2 in response to NAA.

Roots of 5-day-old seedlings harboring *pPLT2:PLT2-GFP* were treated with 1 μM NAA for the indicated times and observed with a confocal microscopy. Bar = 100 μm.

**Figure 7.** Expression of *HPY2* in response to NAA

Confocal microscopy images of root tips of 5-day-old seedlings harboring *pHPY2:HPY2-GFP*, which were treated with (B) or without (A) 1 μM NAA for 12 h. Bar = 100 μm.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A

CDKB2p:GUS/35S:PLT2-GR

0 h

11 h

24 h

B

CDKB2p:CDKB2(m)GUS/35S:PLT2-GR

0 h

11 h

28 h
Figure 6
Figure 7
Supplemental Figure 1

Five-day-old seedlings carrying pCDKB2;1:CDKB2;1(nt)-GUS (CDKB2;1), pCYCB1;1:CYCB1;1-GUS (CYCB1;1) and pCYCB1;2:CYC1;2-GUS (CYCB1;2) were treated with ethanol (control), 10 μM PEO-IAA or 10 μM PEO-IAA plus 100 μM MG132 for 6 h. Root tips were stained for GUS.