Degradation of the ABA co-receptor ABI1 by PUB12/13 U-box E3 ligases

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Clade A protein phosphatase 2Cs (PP2Cs) are abscisic acid (ABA) co-receptors that block ABA signalling by inhibiting the downstream protein kinases. ABA signalling is activated after PP2Cs are inhibited by ABA-bound PYR/PYL/RCAR ABA receptors (PYLs) in Arabidopsis. However, whether these PP2Cs are regulated by other factors remains unknown. Here, we report that ABI1 (ABA-INSENSITIVE 1) can interact with the U-box E3 ligases PUB12 and PUB13, but is ubiquitinated only when it interacts with ABA receptors in an in vitro assay. A mutant form of ABI1-1 that is unable to interact with PYLs is more stable than the wild-type protein. Both ABI1 degradation and all tested ABA responses are reduced in pub12 pub13 mutants compared with the wild type. Introducing the abi1-3 loss-of-function mutation into pub12 pub13 mutant recovers the ABA-insensitive phenotypes of the pub12 pub13 mutant. We thus uncover an important regulatory mechanism for regulating ABI1 levels by PUB12 and PUB13.

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Abscisic acid (ABA) is a plant hormone that regulates seed dormancy, seed germination, seedling growth, as well as biotic and abiotic stress responses. Like other plant hormone signalling pathways, the ABA signalling pathway follows a ‘relief of repression’ model for signal transduction. The clade A protein phosphatase 2Cs (PP2Cs) play a central role in negatively regulating ABA signalling. The cytoplasmic PYR (Pyrabactin Resistance)/PYL (Pyrabactin Resistance 1-Like)/RCAR (Regularly Component of ABA Receptors) ABA receptors (PYLs) bind to ABA and interact with PP2Cs, thereby releasing PP2C inhibition of ABA-activated protein kinases OST1 (SnRK2.6)/SnRK2.2/2.3 (refs 7–9), GHR1 (ref. 10) and SnRK1 (ref. 11), and also some calcium-dependent protein kinases12–14. These protein kinases phosphorylate and activate the key anion channel transcriptional factors to control gene expression in the nucleus; downstream targets such as ABF (ABRE BINDING FACTOR) are involved in the regulation of FLS2 turnover, and PUB13 is also involved in defence response, cell death and flowering. The expression of PUB12 and PUB13 was induced by ABA treatment (Fig. 2b). Histochemical β-glucuronidase (GUS) activity assays indicated that GUS was widely expressed in all tissues including leaves, roots and guard cells in transgenic plants carrying either PUB12 or PUB13 promoter driving GUS (Supplementary Fig. 3). An in vitro ubiquitination assay (Co-IP) assay using proteins extracted from Arabidopsis protoplasts transiently transfected with different plasmids indicated that PUB12-Flag or PUB13-Flag co-ubiquitinated ABI1-Myc but not ABI2-Myc (Fig. 2d). To determine the possibility that ABI1 interacts with PUB12/13 in vivo, we carried out liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis using affinity purified proteins from ProABI1:ABI1-Flag seedlings with anti-Flag antibody. Peptides corresponding to PUB12 were identified in this assay (Supplementary Data 1). We also found that native ABI1 could co-ubiquitinate PUB13-Flag from transgenic plants expressing Pro35S:PUB13-Flag (Supplementary Fig. 5). On the basis of these results we suggest that ABI1 is capable of forming a complex with PUB12 and/or PUB13 and that ABI1 is a receptor protein in the ABA signalling pathway.

Results

ABI1 is degraded by 26S proteasomes. Proteolysis is critical for regulating the turnover of key regulatory proteins in plants. To determine whether ABI1 is regulated by 26S proteasomes, we used immunoblotting to measure the ABI1 level after seedlings were treated with MG132 (an inhibitor of 26S proteasomes). Immunoblotting analysis with anti-ABI1 antibody (see Supplementary Fig. 1 for ABI1 antibody specificity) indicated that ABI1 accumulation was higher in seedlings treated with MG132 than the control (without MG132; Fig. 1a,b). ABA treatment significantly increased ABI1 level comparing without ABA treatment. As ABI1 protein level is very low under normal growth condition, in the next experiments we used the proteins isolated from ABA-treated seedlings. Because ATRP can enhance the protein degradation rate in a cell-free 26S proteasome assay, addition of ATP to total proteins enhanced the degradation rate of ABI1 (Fig. 1c,d). To exclude the translational effect, we treated seedlings with a protein biosynthesis inhibitor cycloheximide (CHX, 100 μM) to block the protein biosynthesis, so that the only changes would be already translated proteins. The results indicated that ABI1 was degraded more quickly with CHX treatment than with MG132 (Fig. 1e,f). These results suggest that the turnover of ABI1 protein is mediated by 26S proteasome pathway.

The U-box E3 ligases PUB12 and PUB13 can interact with ABI1. To determine which E3 ubiquitin ligases target ABI1, we performed yeast two-hybrid assays. We selected the following candidates, which have been shown to be involved in ABA signalling: DWA1 (DWD (CULLIN 4-DAMAGED DNA BINDING 1-DBD1 BINDING WD40) HYPERSENSITIVE TO ABA1), DW2A, RGL1G1/2 (THE MEMBRANE-ASSOCIATED RING DOMAIN LIGASE1/2), SDIR1 (SALT- AND DROUGHT-INDUCED RING FINGER) and KEG (KEEP ON GOING). We also selected some plant U-box E3 ligases (PUBs)5,6,7. The Arabidopsis genome contains 64 genes encoding PUBs, the functions of which are mostly unknown. In total, we tested 29 proteins (including 23 PUB proteins) and found that five proteins (PUB12, PUB13, PUB44, PUB60 and SDIR1) interacted with ABI1 in the yeast two-hybrid assay (Supplementary Fig. 2). Finally, we selected PUB12 and PUB13 for further characterization because these two proteins interacted with ABI1 in both the yeast two-hybrid assay (Fig. 2a) and in other assays, as described later.

PUB12 and PUB13, two highly homologous U-box E3 ligases, are involved in the regulation of FLS2 turnover, and PUB13 is also involved in defence response, cell death and flowering30. The expression of PUB12 and PUB13 was induced by ABA treatment (Fig. 2b). Histochemical β-glucuronidase (GUS) activity assays indicated that GUS was widely expressed in all tissues including leaves, roots and guard cells in transgenic plants carrying either PUB12 or PUB13 promoter driving GUS (Supplementary Fig. 3). An in vitro ubiquitination assay (Co-IP) assay using proteins extracted from Arabidopsis protoplasts transiently transfected with different plasmids indicated that PUB12-Flag or PUB13-Flag co-ubiquitinated ABI1-Myc but not ABI2-Myc (Fig. 2d). To determine the possibility that ABI1 interacts with PUB12/13 in vivo, we carried out liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis using affinity purified proteins from ProABI1:ABI1-Flag seedlings with anti-Flag antibody. Peptides corresponding to PUB12 were identified in this assay (Supplementary Data 1). We also found that native ABI1 could co-ubiquitinate PUB13-Flag from transgenic plants expressing Pro35S:PUB13-Flag (Supplementary Fig. 5). On the basis of these results we suggest that ABI1 is capable of forming a complex with PUB12 and/or PUB13 and that ABI1 is a receptor protein in the ABA signalling pathway.

PUB12/13 mediate ABI1 ubiquitination in vitro. We then used an in vitro ubiquitination assay to test whether PUB12 or PUB13 could ubiquitinate ABI1. All proteins including E1, E2, GST (glutathione S-transferase)-tagged PUB12 (PUB12-GST) or PUB13-GST, ABI1-His and PYR1-GST protein were purified from Escherichia coli, and Flag-tagged ubiquitin (Ub-Flag) is a commercial product. The abi1-1 mutation is hypermorphic, and abi1-1 mutant shows pleiotropic ABA-insensitive phenotypes in all tested ABA responses. The mutation of G180 to D180 in abi1-1 blocks the interaction of ABI1G180-D (ABI1-1) with PYL ABA receptors. The mutated protein ABI1-1-His purified from E. coli was also included in the assays. Consistent with previous results, both PUB12 and PUB13 possessed auto-ubiquitination activity when recombinant E1, E2, Ub-Flag and ATP were added (Fig. 3a,b). Although ABI1-1-His was added to these two reactions
combining with either addition of PYR1 or 5 μM ABA, the ladder-like ubiquitinated ABI1-His could not be detected. Only when both PYR1 and ABA were added together in the ubiquitination reaction, the ladder-like arrangement of proteins with anti-His antibody could be detected, indicating that both PUB12 and PUB13 ubiquitinated ABI1-His (Fig. 3a,b). In contrast, ABI1-1-His was not ubiquitinated in these assays (Fig. 3a,b). We observed that when ABA concentration was increased from $5 \times 10^{-4}$ to 5 μM, the ubiquitination strength of ABI1-His was gradually increased (Fig. 3c), suggesting that ABI1 ubiquitination relies on ABA concentration in presence of PYR1.

PYL ABA receptors can be divided into two subgroups according to their interaction with PP2Cs. One group includes PYR1 and PYL1-3 that interact with and inhibit PP2Cs only after they bind to ABA. The other group includes PYL4-10 that can interact with and inhibit PP2Cs without binding to ABA, but their inhibition of PP2Cs is stronger after they bind to ABA17. We selected PYL4 and PYL9 from the latter group to determine whether ABI1 can be ubiquitinated by PUB13 when either PYL4 or PYL9 is available in the in vitro ubiquitination assay using proteins purified from E. coli as performed above. Immunoblotting analysis with anti-His antibody revealed that ABI1-His could be ubiquitinated with or without ABA (5 μM) in the presence of PYL4-GST or PYL9-GST in the ubiquitination assays (Fig. 3d). However, the ubiquitination levels were slightly higher with addition of ABA than without ABA. PYR1 with or without addition of ABA (5 μM) was used as controls. These results suggest that PUB13-mediated ABI1 ubiquitination depends on the interaction of ABI1 with ABA receptors in the in vitro assay. PUB13-mediated ABI1 ubiquitination in presence of PYL4 and PYL9 without ABA suggests that ABI1 may be also dynamically regulate at protein level even under normal conditions.

PUB12/13 are required for ABI1 degradation. To determine whether PUB12 and PUB13 modulate ABI1 degradation in plant cells, we compared ABI1 protein level between pub12 pub13 mutant and the wild type using anti-ABI1 antibody. A previous study indicated that the transcription of PUB12 in pub12 (pub12-2 mutant) is significantly reduced and pub13 is a null mutant allele29. Immuno blotting analysis indicated that more ABI1 accumulated in the pub12 pub13 mutant than in the wild type with or without ABA treatment (Fig. 4a). As ABI1 transcripts were lower in the pub12 pub13 mutant than in the wild type, but higher than abi1-1 (Col) (the same mutation as the abi1-1 in Ler)35 (Fig. 4b, see also RNA-seq data in Supplementary Data 2 and 3), the higher accumulation of ABI1 protein in the pub12 pub13 mutant than the wild type may be attributed to post-transcriptional regulation. In order to examine the effect of PUB12/13 on ABI1 protein degradation in plants, we treated seedlings with 100 μM CHX to block protein translation and then performed an immunoblotting assay with anti-ABI1 antibody. As shown in Fig. 4c,d, the degradation of ABI1 protein occurred more slowly in the pub12 pub13 mutant than wild type.

To test the effect of increasing PUB13 on ABI1 stability in plant cells, we transiently co-transfected transgenic Pro35S:PYR1-Flag Arabidopsis protoplasts with Pro35S:ABI1-Myc plus increasing amount of Pro35S:PUB13-Flag plasmids (Fig. 4e). Here Pro35S:PYR1-Flag transgenic plants were used as we consider that more PYR1 proteins are required when more ABI1 proteins are expressed in this assay. After the protoplasts were cultured for 16 h, and then treated with or without 10 μM ABA for 4 h, the total proteins were extracted and used for immunoblotting analysis using anti-Myc antibody. ABI1-Myc protein level gradually decreased with increasing PUB13-Flag (Fig. 4e, left). However, when the protoplasts were not treated with ABA, ABI1-Myc protein level was not obviously changed (Fig. 4e, middle). We also co-transfected Pro35S:ABI1-Myc plasmids and a
mutated form PYR1 plasmids plus increasing amount of Pro35S:PUB13-Flag plasmids into protoplasts. The mutated PYR1\textsuperscript{PS8} does not interact with ABI1 in the presence of ABA\textsuperscript{7}. We did not observe a clear reduction of ABI1-Myc protein in the assay (Fig. 4e, right). These results suggest that PUB13 may promote ABI1-Myc degradation depending on both the presence of ABA and interaction with PYR1 in transgenic Pro35S:PYR1-Flag Arabidopsis cells. Although ABI1 and ABA-bound PYR1 form a stable complex, PYR1-Flag was not degraded by PUB13 in the assay (Fig. 4e). However, PYR1 must be degraded by other E3 ligases because PYR1 degradation also depends on the 26S proteasome (Supplementary Fig. 6). Consistently, PYR1/PYL4 and PYL8 have been shown to be degraded by a single subunit RING-type E3 ubiquitin ligase RSL1 (RING FINGER OF SEED LONGEVITY1) and a DDA1 (DET1-, DDB1-ASSOCIATED1) E3 ligase, respectively\textsuperscript{36,37}.

To determine whether ABI1 can be ubiquitinated in plant cells, we used a P62-agarose matrix that is capable of binding ubiquitinated proteins to enrich the ubiquitinated proteins from two independent transgenic seedlings stably expressing Pro35S:ABI1-Myc or from wild-type plants as a negative control. The bound proteins were used for immunoblotting analysis with anti-Myc antibody. As shown in Fig. 4f, the ladder-like protein pattern was detected in the enriched proteins from two transgenic plants but not from wild-type plants. This result indicates that ABI1 can be ubiquitinated in plant cells.

**ABA promotes ABI1 degradation in plants.** As ABA is absolutely required for the interaction of ABI1 and PYR1 in order for PUB12/13 to ubiquitinate ABI1 in the \textit{in vitro} assay (Fig. 3a,b), we asked whether ABA influences ABI1 degradation in plants. The wild-type seedlings were treated with 100 \textmu M CHX or treated with 100 \textmu M CHX plus 50 \textmu M ABA for 0, 1, 2 and 3 h, and then the total proteins were used for immunoblotting with anti-ABI1 antibody. ABA treatment reduced the ABI1 protein level more than the control treatment (Fig. 5a,b). We also purified ABI1-His from an ABA-deficient mutant aba2-21 (containing < 10% ABA of the wild type)\textsuperscript{38} in presence of ATP. ABI1-His was more degraded in the extracts from the wild type than aba2-21.
ABI1 was ubiquitinated with addition of PYL4 or PYL9. However, addition of ABA slightly increases ubiquitination level compared with no addition of ABA. Ubiquitination was detected by anti-His antibody, and the activity of PUB13 E3 ligase was detected by anti-Flag antibody. Whether or not ABA was added, ubiquitines ABI1 in presence of PYL4 and PYL9 with or without addition of ABA. Different proteins purified from respectively. ABI1 ubiquitination was detected with anti-His antibody, and the overall ubiquitination was detected with anti-Flag antibody. (pretreated with 50 μM ABA) or without ABA. PYL4 and PYL9 interact with ABI1 in the absence of ABA, while PYR1 interacts with ABI1 only in the presence of ABA. ABI1 ubiquitination was detected by anti-His antibody, and the activity of PUB13 E3 ligase was detected by anti-Flag antibody. Whether or not ABA was added, ABI1 was ubiquitinated with addition of PYL4 or PYL9. However, addition of ABA slightly increases ubiquitination level compared with no addition of ABA.

Figure 3 | PUB12 and PUB13 ubiquitinate ABI1 in an in vitro assay. (a,b) PUB12 and PUB13 ubiquitinate ABI1 depending on the addition of both PYR1 and ABA in the assays. Different proteins purified from E. coli were added to the ubiquitination reaction buffer with 5 μM ABA or without ABA. The hypermorphic mutation ABI1-1 (ABI1G180D) protein was included in the reaction. ABI1 ubiquitination was detected with anti-His antibody, and the overall ubiquitination was detected with anti-Flag antibody. (c) Increasing ABA concentration increases ubiquitinated ABI1 level. The same amount of different proteins (here–PUB13 was used as an E3 ligase) were included in the ubiquitination reactions with addition of 0, 5 × 10⁻⁴, 5 × 10⁻² or 5 μM ABA, respectively. ABI1 ubiquitination was detected with anti-His antibody, and the overall ubiquitination was detected with anti-Flag antibody. (d) PUB13 ubiquitates ABI1 in presence of PYL4 and PYL9 with or without addition of ABA. Different proteins purified from E. coli were added to ubiquitination buffer with 5 μM ABA or without ABA. PYL4 and PYL9 interact with ABI1 in the absence of ABA, while PYR1 interacts with ABI1 only in the presence of ABA. ABI1 ubiquitination was detected by anti-His antibody, and the activity of PUB13 E3 ligase was detected by anti-Flag antibody. Whether or not ABA was added, ABI1 was ubiquitinated with addition of PYL4 or PYL9. However, addition of ABA slightly increases ubiquitination level compared with no addition of ABA.

Figure 4 | PUB12/13 are required for ABI1 degradation in plant cells. (a) ABI1 level is higher in the pub12 pub13 double mutant than in the wild type. The total proteins extracted from wild-type plants or the pub12 pub13 mutant treated with or without 50 μM ABA for 6 h were used for immunoblotting analysis with anti-ABI1 antibody. Total proteins from the pub12 pub13 mutant were diluted from 100 to 50 μg, and short and long exposure time were used in order for comparison. ACTIN was used as a loading control. (b) Relative expression of ABI1 in the wild type (Col), the pub12 pub13 mutant and abi1-1 (Col). Total RNAs extracted from 7-day-old seedlings treated with 50 μM ABA for 1 h were used for real-time RT-PCR. (c) Comparison of degradation between the pub12 pub13 mutant and the wild type. The 7-day-old seedlings were treated with 100 μM CHX for different times. At each time point, total proteins were extracted and used for immunoblotting analysis with anti-ABI1 antibody. ACTIN was used as a loading control. (d) Quantitative analysis of the signal intensity in e. The abundance of ABI1 at the 0 h was set to 1 as a reference for calculating relative abundance of various time point. Error bars are means ± s.e.m. (n = 3 independent experiments). (e) PUB13-mediated ABI1 degradation requires ABA as well as ABI1 interaction with PYR1. The protoplasts from a transgenic line overexpressing PYR1-Flag or the wild type seedling were co-expressed with 10 μg of pro35S:ABI1-Myc and different amounts of pro35S:PUB13-Flag plasmids (0 to 200 μg) or together with 10 μg of Pro35S:PYR1P88S (only for wild type protoplasts) for 16 h and treated with 10 μM ABA (left, right) or without ABA (middle) for 4 h before immunoblotting analysis was performed using anti-Myc antibody or anti-Flag antibody. PYR1-Flag was used as the loading control. (f) ABI1 is ubiquitinated in plants. Ubiquitinated proteins were enriched from P62-agarose matrix that was incubated with total proteins isolated from two independent transgenic plants stably expressing ABI1-Myc or from wild-type plants. Plant materials were pretreated with 50 μM ABA for 12 h and 50 μM MG132 for 6 h before immunoblotting analysis was performed using anti-Myc antibody.

in these cell-free 26S proteasome assays (Supplementary Fig. 7). These results suggest that ABI1 degradation is promoted by ABA.

ABA receptors are required for ABI1 degradation. If the interaction of ABI1 and PYLs is a prerequisite for PUB12/13-mediated ubiquitination of ABI1, then we hypothesized that
Figure 5 | ABI1 degradation is promoted by ABA or ABA receptors in plants, and delayed by ABI1-1 dominant mutation. (a) ABA promotes ABI1 degradation. The 7-day-old wild type seedlings were treated with 100 μM CHX or 100 μM CHX plus 50 μM ABA for different times. At each time point, proteins were extracted and used for immunoblotting analysis. ACTIN was used as a loading control. (b) Quantitative analysis of the signal intensity in a. The abundance of ABI1 at the 0 h (CHX, CHX + ABA) was set to 1 as a reference for calculating relative abundance of various time point. Error bars are means ± s.e.m. (n = 3 independent experiments). (c) ABI1 protein is more stabilized in pyl1 pyl2 pyl4 quadruple mutant than in the wild type. The total extracted proteins were extracted from 7-day-old seedlings treated with 100 μM CHX for different times and used for immunoblotting analysis with anti-ABI1 antibody. ACTIN was used as a loading control. (d) Quantitative analysis of the signal intensity in c. The abundance of ABI1 at the Col 0 h was set to 1 as a reference for calculating relative abundance of various time point. Error bars are means ± s.e.m. (n = 3 independent experiments). (e) ABI1 protein is more stable in abi1-1 than in the wild type. The total extracted proteins from the 7-day-old wild type or abi1-1 (Col) seedlings treated with 100 μM CHX for different times were used for immunoblotting analysis with anti-ABI1 antibody. ACTIN was used as a loading control. (f) Quantitative analysis of the signal intensity in e. The abundance of ABI1 at the Col 0 h was set to 1 as a reference for calculating relative abundance of various time point. Error bars are means ± s.e.m. (n = 3 independent experiments).

To this end, total proteins were extracted from PYL quadruple mutants (p’y1 pyl1 pyl2 pyl4)7 or the wild-type plants treated with 100 μM CHX for 0, 1, 3 and 6 h, and used for immunoblotting with anti-ABI1 antibody. As shown in Fig. 5c,d, ABI1 protein level was lower in the PYL quadruple mutant than the wild type, but the degradation of ABI1 was much slower in the quadruple mutant than in the wild type. Moreover, ABI1 protein level induced by ABA was much less in the quadruple mutant than in the wild type (Supplementary Fig. 8a). We further performed a cell-free protein degradation assay by combining total proteins extracted from the wild type or the quadruple mutant with ABI1-His protein purified from E. coli in presence of ATP. The results showed that the degradation of ABI1-His was slower in protein extraction from the quadruple mutant than from the wild type (Supplementary Fig. 8b). On the basis of these results we conclude that ABI1 degradation requires ABA receptors.

As the mutation in ABI1-1 blocks its interaction with ABA receptors, ABI1-1 is not ubiquitinated in the in vitro assay (Fig. 3a,b). We next examined ABI1-1 stability in plant cells by determining the ABI1 level with anti-ABI1 antibody. Immunoblotting analysis indicated that ABI1 was accumulated more under normal growth condition, but less with ABA treatment in abi1-1 mutant than the wild type (Supplementary Fig. 9a). After seedlings were treated with 100 μM CHX for different times, ABI1 protein level was checked. ABI1 protein was gradually reduced in the wild type, but only reduced to a certain level in abi1-1 (Col) as time went on (Fig. 5e,f). In addition, we transiently transfected ABI1-1-Myc or ABI1-Myc plasmids, respectively into Arabidopsis protoplasts. After the protoplasts were cultured for 14 h, total proteins were extracted from these protoplasts and used for the cell-free protein degradation assay. The degradation of ABI1 wild-type protein was greatly enhanced compared with mutated ABI1-1 in presence of ATP (Supplementary Fig. 9b). These results imply that the reduced ABI1-1 degradation is likely due to its failed interaction with ABA-bound PYLs.

PUB12/13 are involved in the ABA signalling pathway. Because PUB12/13 target ABI1 for its degradation and because the pub12 pub13 mutant greatly reduces ABI1 degradation compared with the wild type, we speculated that the pub12 pub13 mutant would reduce the ABA response. SnRK2.2/2.3/2.6 are specially inhibited by clade A PP2Cs6,7 and ABA-activated OST1/SnRK2.6 is one of the most important outputs in ABA signalling. An in-gel assay using total proteins extracted from seedlings treated with ABA indicated that protein kinase activity corresponding to OST1 (OST1 shows the highest activity among SnRK2.2, SnRK2.3 and SnRK2.6/OST1) was lower in pub12 pub13 mutant than in the wild type, but higher than in the abi1-1 (Col) (Fig. 6a,b). The ost1-3 mutant was used as a negative control. These results suggest that the reduced OST1 kinase activity results from increased activity of PP2Cs, most likely due to accumulation of ABI1 in the pub12 pub13 mutant. As expected, pub12 and pub13 were more resistant to ABA than wild type when cotyledon greening after seed germination was examined (cotyledons become green after seed germination; Fig. 6c,d). The pub12 pub13 double mutant showed an enhanced ABA-insensitivity phenotype in cotyledon greening relative to pub12 or pub13 (Fig. 6c,d). pub12, pub13 and pub12 pub13 were also more resistant to inhibition of root growth by ABA than the wild type (Fig. 6e,f). However, the ABA-resistant cotyledon greening and root growth phenotypes were much weaker in the pub12 pub13 mutant than in abi1-1 (Col)45,35. A recent study suggests that ABI1 is a negative mediator in plant freezing tolerance39. Consistently, pub12 pub13 seedlings showed increased sensitivity and ion leakage to freezing stress under both non-acclimated and cold-acclimated conditions compared with the wild type (Supplementary Fig. 10).
As pub12 pub13 mutant accumulates more ABI1 protein than the wild type, we expected that pub12 pub13 could regulate the expression of some common genes as abi1-1 (Col) under ABA treatment conditions. Ten-day-old seedlings were treated with 50 μM ABA for 0, 1 and 3 h. Total RNAs were isolated and used for RNA-deep sequencing on Illumina Hiseq platform. The 125 bp trimmed paired-end reads with high quality were generated and mapped to the Arabidopsis genome (TAIR10) using TopHat (http://tophat.cbcb.umd.edu/) with default settings.90. RNA-seq data were collected from two independent experiments (each sample with 2.0 G clean data) and differential gene expression analysis was performed using Cufflinks (http://cufflinks.cbcb.umd.edu/)11. These analyses identified 3,580 and 4,225 genes that were significantly induced by 50 μM ABA at 1 h and 3 h, respectively, in the wild type (Supplementary Data 2 and 3). We then compared the expression levels of these ABA-induced genes in the wild type with those in pub12 pub13 and abi1-1 (Col). Among 3580 ABA-induced genes at 1 h, the expression levels of 2,237 genes were lower in pub12 pub13, and 2,024 genes lower in abi1-1 (Col) than the wild type (Fig. 6g; Supplementary Data 2 and 3). The expression levels of 1,327 genes were lower in both pub12 pub13 and abi1-1 (Col) than in the wild type. Similarly, among 4,225 ABA-induced genes at 3 h, 2,724 genes in pub12 pub13 and 2,679 genes in abi1-1 (Col) were downregulated, and 1,972 genes were downregulated in both pub12 pub13 and abi1-1 (Col) compared with the wild type (Fig. 6g). The expression levels of ABA-induced marker genes such as RD29A (AT5G52310), KIN1 (AT5G01520), ABI1 (AT4G26080), ABI2 (AT1G72770), HAB1 (AT1G17550), and HAB2 (AT1G17550) in pub12 pub13 were higher than in abi1-1 (Col), but lower than in the wild type (Supplementary Data 2 and 3). Heat map analysis indicates that the expression of ABA-induced genes has a strong correlation between pub12 pub13 and abi1-1 (Col) (Fig. 6g), which implies that pub12 pub13 mutant reduces ABA signalling likely through promoting accumulation of ABI1 protein.

ABA promotes the production of H₂O₂ in guard cells42,43. In the ABA signalling pathway, ABI1 acts as a negative factor upstream of H₂O₂ to mediate stomatal movement44. The abi1-1 mutation greatly reduces H₂O₂ production in guard cells44. We expected that the mutations in pub12 pub13 would cause ABI1 to accumulate, which would result in the reduced accumulation of H₂O₂ in guard cells. In the absence of ABA, the guard cells of pub12 pub13 produced less H₂O₂ than those of the wild type or the pub12 or pub13 mutant (Fig. 7a,b). ABA treatment significantly increased H₂O₂ production in the guard cells of the wild type but significantly decreased H₂O₂ production in guard cells of pub12, pub13 or pub12 pub13 (Fig. 7a,b). These
A representative experiment; three independent experiments were done with similar results. Values are means ± s.d. of three replicates (30 stomata from one seedling in each replicate) from one representative experiment; three independent experiments were done with similar results. Values are means ± s.d. of three replicates (40 leaves from one pot were measured per replicate) from one representative experiment. pub12 pub13 mutations impair ABA-induced stomatal closure. Leaf epidermal peels were treated with MES buffer for 2 h under strong light to fully open stomata. After the peels were incubated with different concentrations of ABA for 2 h, stomatal apertures were measured with Image J. Values are means ± s.d. of three replicates (120–150 stomata from one seedling in each replicate) from one representative experiment. (e) pub12 pub13 mutants lose more water and are more sensitive to drought stress than the wild type (Fig. 7d). Using isolated epidermal peels, we found that ABA-induced stomatal closure (Fig. 7e) and ABA-inhibited stomatal opening (Fig. 7f) were impaired in pub12, pub13 and pub12 pub13 mutants. These results indicate that PUB12 and PUB13 are involved in ABA-mediated stomatal movement.

abi1-3 recovers ABA-insensitivity of pub12 pub13. The above results suggest that PUB12/13 target ABI1 for its degradation. If this is the case, genetically, ABI1 should act downstream of PUB12/13, and abi1 loss-of-function mutant should block ABA-insensitive phenotypes of pub12 pub13 mutant. In order to test this hypothesis, we introduced the abi1-3 loss-of-function allele into pub12 pub13 mutant by crossing abi1-3 (a T-DNA insertion mutant, Supplementary Fig. 1 for ABI1 protein level)15 with pub12 pub13 and tested the ABA response of the abi1-3 pub12 pub13 triple mutant. Previous studies show that abi1 loss-of-function mutant does not show any apparent ABA phenotype compared with the wild type as these PP2Cs are redundant in ABA signalling5,46. We first compared the root growth of the abi1-3 pub12 pub13 triple mutant with the pub12 pub13 double mutant and abi1-3 with ABA treatment. As shown in Fig. 8a,b, the abi1-3 pub12 pub13 triple mutant showed similar root growth phenotype as abi1-3 or the wild type with ABA treatment. The root growth of pub12 pub13 was more resistant to ABA than the triple mutant, abi1-3 or the wild type. We further compared the ROS production. abi1-3 pub12 pub13 triple mutants produced similar amount of ROS as abi1-3, but significantly more than pub12 pub13 without or with ABA treatment (Fig. 8c).

Furthermore, abi1-3 pub12 pub13 triple mutant exhibited similar ABA-induced stomatal closure (Fig. 8d) and ABA-inhibited stomatal opening (Fig. 8e) as abi1-3 or the wild type, indicating that ABI1 loss function recovers the impairment of ABA-regulated stomatal movement in pub12 pub13. In detached-leaf water loss, abi1-3 pub12 pub13 triple mutant lost similar water as abi1-3, but less than pub12 pub13 (Fig. 8f). All these genetic data indicate that PUB12/13 act upstream of ABI1 to modulate ABA response.

Discussion
PP2Cs are key repressors in the ABA signalling pathway. The ABA receptors PYLs bind to ABA, which allows the capture of PP2C proteins and the inhibition of PP2C activity. The entire ABA signalling pathway in Arabidopsis can be reconstituted in vitro by co-expression of ABA signalling core components including PYLs, PP2Cs, SnRKs and ABF2 (ref. 47), suggesting that the PP2C inhibition by ABA-bound PYLs is sufficient to...
activate SnRKs. Previous studies indicate that ABI1 is upregulated at transcriptional level by ABA in a negative feedback regulatory loop\(^6\). In this study, we found that when interacting with ABA receptors, ABI1 is degraded by the 26S proteasome pathway, which consequently enhances ABA signalling (Fig. 9). These results suggest that both the inhibition and degradation of ABI1 are important for activating ABA signalling in vivo. Our results suggest that PUB12/13 can interact with ABI1, but it may be ubiquitinated ABI1 only when ABI1 interacts with PLYs both with ABA (such as with PYR1) and without ABA (such as with PLY4/9) in the in vitro assays (Fig. 3a,b). However, ABA apparently promotes the degradation of ABI1 (Fig. 5a; Supplementary Fig. 7). It is possible that interaction of ABI1 with PLYs changes the conformation of ABI1, which may create a suitable surface for ubiquitin transfer.

Although the degradation of ABI1 in a PYL quadruple mutant is largely reduced (Fig. 5), ABI1 protein level is lower in the PYL quadruple mutant than in the wild type under both ABA treatment and control treatment (that is, normal growth condition), consistent with previous studies that show ABA signalling is reduced by PYL mutations\(^9\). Interestingly, ABI1 protein is even higher in abi1-1 mutant than in the wild type with no ABA treatment (under normal growth condition), but lower with ABA treatment. As the ABI1-1 mutation is hypermorphic, ABI1-1 protein is even higher in the triple mutant than in the wild type under normal condition.

Figure 8 | abi1-3 loss-of-function mutant recovers the ABA-insensitive phenotypes of pub12 pub13. (a) The abi1-3 pub12 pub13 triple mutant is similar to abi1-3 or wild type, but more sensitive to ABA than pub12 pub13 mutant. (b) Statistical analysis of ABA-inhibited root growth in a. Root length is relative to the control (without ABA). Three independent experiments were conducted, each with three replicates. Values are means ± s.d., n = 3 (**\(P<0.01\), Student’s t-test). (c) ABA-induced H₂O₂ accumulation in guard cells is comparable among abi1-3, abi1-3 pub12 pub13 triple mutant and the wild type that produces more H₂O₂ than the pub12 pub13 double mutant. H₂O₂ accumulation was assessed by H2DCF-DA staining following treatment with 0 or 50 μM ABA for 5 min. Fluorescence intensity is relative to the wild type without ABA treatment. Values are means ± s.d. of three replicates (30 stomata from one seedling in each replicate) from one representative experiment; three independent experiments were done with similar results (**\(P<0.01\), Student’s t-test). (d) The abi1-3 pub12 pub13 triple mutant recovers the impaired ABA-induced stomatal closure. The same treatment was done as in Fig. 7e. Values are means ± s.d. of three replicates (120–150 stomata from one seedling in each replicate) from one representative experiment; three independent experiments were done with similar results (**\(P<0.01\), Student’s t-test). (e) The abi1-3 pub12 pub13 triple mutant recovers the impaired ABA-inhibited stomatal opening of pub12 pub13. The same treatment was done as in Fig. 7f. Values are means ± s.d. of three replicates (120–150 stomata from one seedling in each replicate) from one representative experiment; three independent experiments were done with similar results (**\(P<0.01\), Student’s t-test). (f) Water loss from detached leaves is similar in the abi1-3 pub12 pub13 triple mutant and abi1-3 that lose less water than pub12 pub13. Values are means ± s.d. of three replicates (40 leaves from one pot were measured per replicate) from one representative experiment.
The stronger ABA-insensitive phenotypes of those of abi1-1 constitutively respond to ABA. Not clearly differ in ABA response and that PP2C triple mutants observations that phenotypes between PUB13-overexpressing plants and the wild pub12 pub13 mutant, ABI1 protein can still be accumulated under stress conditions, ubiquitination and degradation of ABI1 after it interacts with PYR1 (with ABA) or PYL9 (with and without ABA). The transcripts of ABI1 and other related PP2Cs are induced by ABA signaling. ABI1 interacts with and inhibits OST1. ABI1 can also interact with PUB12/13. After ABA-bound PYR1 interacts with ABI1, protein kinases such as OST1 are released and activated to phosphorylate downstream targets including transcriptional factors (TFs) and SLAC1 in guard cells. PUB12/13 are able to ubiquitinate ABI1 likely because of the conformational change of ABI1 after it interacts with PYR1 (with ABA) or PYL9 (with and without ABA). The ubiquitinated ABI1 is degraded by 26S proteasomes. The interaction of the GA receptor GID1 with DELLA proteins is promoted by the binding of GA to GID1. The GID1-GA-DELLA complex facilitates the interaction of the DELLA C terminus with the F-box protein GID2-based SCF/GID2 complex in rice (SLEEPY 1 [SLY1]-based SCF/SLY1 in Arabidopsis), and DELLA2s are in turn ubiquitinated and degraded through the 26S proteasome pathway. The GID1-GA-DELLA complex would also reduce the availability of DELLA for interacting with and inhibiting its target transcriptional factor. Like ABA and GA signaling, the signaling for other phytohormones such as auxin, jasmonate and strigolactone also follows a ‘Relief of Repression’ module that degrades the negative regulators via receptor-mediated proteolysis. These results suggest that plants have evolved similar regulatory mechanisms in hormone signaling so as to quickly respond to environmental challenges under natural conditions.

Methods

Plant materials and growth conditions. Arabidopsis thaliana (Col-0 accession) seeds were sown on MS medium containing 2% sucrose and 0.8% agar. At 5–7 days after germination, seedlings were transferred to soil and grown under short-day (12-h light/12-h dark) or long-day (16-h light/8-h dark) conditions in a growth room at 20–22°C. The T-DNA insertion mutants used in this study were pub12 (salk_093164) and pub12 (Wisc006997_01). Overexpression transgenic plants, the cDNAs of ABI1, PUB12 and PUB13 were amplified and cloned into the pCAMBIA1300 vector under the 35S promoter. The correct clones were transformed into Agrobacterium tumefaciens strain GV3101 and transferred into Arabidopsis plants (wild type and the pub12 pub13 double mutant) by floral dip method. Twenty T3 homozygous transgenic lines were screened, and at least two lines were used for experiments. The primers used for identification of the mutations and for construction of transgenic plants are listed in Supplementary Table 1.

Drought-related phenotype analyses. For a water loss assay with detached leaves, rosette leaves were cut from Col-0, abi1-3, pub12, pub12 pub13, abi1-3 pub12 pub13 plants grown in soil under normal short-day conditions in a growth room. The detached leaves were weighed, placed on a piece of weighing paper in a growth room (20°C and 75% humidity), and periodically weighed every hour for at least 6 h. Water loss was expressed as a percentage of the original fresh weight of the detached leaves. The experiment was independently repeated twice.

For stomatal aperture measurement, epidermal strips were peeled from rosette leaves of 4-week-old seedlings. The chlorophyll on the epidermal strips was removed with a writing brush. The epidermal strips were then immersed in opening solution MES buffer (10 mM MES-KOH (pH 6.15), 10 mM KCN and 50 μM CaCl2) under light (90 μmol m−2 s−1) for 2 h at 22°C. The treated epidermal strips were then transferred to MES buffer containing 0, 1 or 5 μM ABA.
After incubation for 2 h in light, the epidermal strips were photographed with an OLYMPUS RX53 microscope and were measured with Image J 1.47V software. For an assay assessing ABA inhibition of stomatal opening under light, 4-week-old plants were cultured in darkness for 24 h to make the stomata close. Then, epidermal strips were quickly peeled and immersed under light for 5 min, and the fluorescent in guard cells was detected with a confocal microscope (OLYMPUS BX53) under 514 nm excitation and 530 nm emission. The fluorescence intensities were analyzed using AxioVisionRel. 4.8 software. About 30 guard cells were assessed per sample, and the experiment was independently performed three times.

**Gene expression analysis by quantitative RT–PCR.** Total RNAs were extracted from these seedlings with TRIzol reagent (Life Technologies, cat. no. 15956-018). A 4-μg quantity of DNase I-treated total RNAs was used as template for first-strand cDNA synthesis by M-MLV reverse transcriptase (Promega, cat. no. M170A). cDNAs were diluted 10 times with ddH2O, and 2 μl was used for PCR. Quantitative PCR with reverse transcription (qRT–PCR) was performed with SYBR premix ExTaq (TaKaRa, cat. no. RR820A) and with gene-specific primers and the internal control (Actin4). The primers used for qRT–PCR are listed in Supplementary Table 1. The reactions were run on a 7300 Real-Time PCR system. The reaction conditions included 40 cycles at 95 °C for 5 min, 95 °C for 1 s, and 60 °C for 34 s. The primers used for qRT–PCR are listed in Supplementary Table 1.

**Purification of ubiquitinated proteins.** Wild-type and two Pro35S:ABI1-Myc transgenic plants were grown in MS medium for 10 days and were then treated with 50 μM ABA for 12 h and 50 μM MG132 for 6 h. Total proteins were extracted with 1 ml of BI buffer (50 mM Tris-Cl, pH 7.5), 20 mM NaCl, 0.1% NP-40 and 5 mM ATP) in a prechilled mortar. The following were added to the protein homogenates: 1 mM PMSF, 0.1 mM MG132, 10 mM Ub aldehyde (Sigma-Aldrich, cat. no. SRP6024), and 10 mM N-ethylmaleimide (Sigma-Aldrich, cat. no. E1271). After proteins were purified, 2 μg of total proteins in a total volume of 2 μl was used for the assay. An 80 μl volume of protein supernatants was reserved as input. Other protein supernatants were incubated with 40 μl of prewashed p62-agarose (Enzo Life Sciences, cat. no. BML-UW9010-0500) in 2 ml of BI buffer at 4 °C. After 4 h, the agaroses were washed twice with BI buffer and once with BI buffer (supplemented with 200 μM NaCl in BI). Samples were boiled in 50 μl of 1 × SDS loading buffer for 5 min. The ubiquitinated proteins were separated by 10% SDS–PAGE gel, and anti-Myc antibody was used to detect ubiquitinated ABI1-Myc protein. ACTIN was used as an equal loading control.

**Yeast two-hybrid assay.** To confirm the interaction between ABI1 and PUB12/13, and between ABI1 and other proteins, full-length PUB12/13 or other genes and ABI1 cDNA were separately fused into pGBK7 (binding domain, BD) and pGADT7 (activation domain, AD) vectors. These plasmids were co-transformed into yeast strain AH109. Transformed yeast cells were selected on SD dropout medium (–Trp– Leu) and 3% Dextrose medium (–Trp– Leu– His) and incubated at 28 °C for 4–5 days. If the proteins in BD vector exhibited self-activation, 30 mM 3-AT (3-amino-1, 2, 4-triazole) was added to suppress the self-activation.

**High-throughput mRNA sequencing analyses.** Ten-day-old seedlings grown on MS medium in the plastic plates under 23 h light/1 h dark at 22 °C were treated with 1 μM ABA for 0, 1, and 3 h. Total RNA was isolated with Nano easy RNA Kit (QIAGEN, cat. no. 74904) according to the kit instructions. Three microgram RNAs for each treatment were used for library construction and RNA-seq on Illumina HiSeq 2500 platform. The libraries were constructed using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA, cat. no. E7420L) following the instructions.

About 2.0-GB clean reads were generated for each sample (number of reads per sample and alignment statistics were listed in Supplementary Table 2). All reads were trimmed to 125 bp paired-end reads with high quality according to the base quality. (Original raw read length were about 250 bp, 125 bp paired-end clean reads were generated, and the quality of each base was evaluated by the base quality Q<sub>30</sub>≥5 was >50% for one read, the paired-end reads were discarded.) The trimmed reads were mapped to the genome of A. thaliana (TAIR10) using TopHat (http://ccb.jhu.edu/software/tophat/index.shtml) with default settings. Differential gene expression analysis was performed using Cufflinks (http://cole-trapnell-lab.github.io/cufflinks) with each pair of reads as input. The reads mapped reads was used to indicate the gene expression level. Differentially expressed genes were selected by the comparison of gene expression levels of the
control sample (treated 0 h with ABA) with treatment samples (1 or 3 h with ABA) for the wild type (Col), pub12 pub13 and abi1-1 (Col), respectively (using Student’s t-test with P < 0.01 and q < 0.05). Genes significantly induced by ABA in the Col group were chosen for the comparison with expression levels of the treatment samples between different groups (Supplementary Data 2 and 3). Fold changes of the genes induced significantly by ABA treatment were compared with the control sample in each group. To compare the change levels of the treatment samples between different groups, we calculated relative expression level. Fold change of each gene in the treatment samples in pub12 pub13 or abi1-1 minus the fold change of the same gene in the treatment samples in Col was considered as the relative expression level of the gene in pub12 pub13 or abi1-1 comparing to Col. Thus positive or negative. When the relative expression level indicated the change level of the gene in pub12 pub13 or abi1-1 was higher or lower than the change level in Col. For purposes of presentation we multiplied the relative expression level by 5, and we considered multiplied relative expression level of less than – 10 as – 10. We then drew the heat maps based on the multiplied relative expression levels using heatmap.2 function in the gplots package in R. Complete linkage hierarchical clustering with Euclidean distance as a distance measure was used to sort the rows. Other antibodies used for Immunoblotting assay were listed in Supplementary Table 3. All original immunoblots are provided in Supplementary Fig. 12.

**LC-MS/MS analysis.** To detect the interaction proteins with ABI1, we performed LC–MS/MS assay using ProAB11ABI1-Flag transgenic lines. Fifteen grams of transgenic plant seedlings were collected and grinded in liquid nitrogen. The powdered grid was dissolved in lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 100 mM EDTA, 0.2% SDS, 0.5% NP-40, adding 1 mM PMSF protease inhibitor and 1 mM DTT before using). Then the samples were placed on ice for 10 min and centrifuged at 4,000g for 4 min. Supernatant was transferred to a new tube, and centrifuged at 12,000g for 4 min. Supernatant was transferred to a new tube containing 300 µl Flag beads, incubated at 4 °C for 2 h with slowly shaking. After 300 µl centrifuged at 4 °C for 10 min, Flag beads were collected and washed with lysis buffer for 3–5 times. A volume of 300 µl elution buffer (containing 100–500 µg ml⁻¹ Flag peptide in PBS buffer) was added to Flag beads, rotated at 4 °C for 1 h and repeated three times. All of the elution buffer (total about 900 µl) was collected together and concentrated with ultra-filtration column (Millipore, cat. no. UK510024) to 50 µl for the final sample. The sample was separated in 10% SDS–PAGE gel and digested with trypsin. Digested peptides were performed on a Thermo Q-Exactive high resolution mass spectrometer (Thermo Scientific, Waltham, MA, USA). Source parameters were 2 kV spray voltage and 320 °C capillary temperature. Data obtained from the mass spectrometer were preprocessed with Mascot Distiller 2.4 for peak picking. The resulted peak lists were searched against Swissprot database using Mascot 2.4 search engine.

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