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Research

The *Arabidopsis* E3 ubiquitin ligase DOA10A promotes localization of abscisic acid (ABA) receptors to the membrane through mono-ubiquitination in ABA signaling

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Summary

- The endoplasmic reticulum-associated degradation (ERAD) system eliminates misfolded and short-lived proteins to maintain physiological homeostasis in the cell. We have previously reported that ERAD is involved in salt tolerance in *Arabidopsis*. Given the central role of the phytohormone abscisic acid (ABA) in plant stress responses, we sought to identify potential intersections between the ABA and the ERAD pathways in plant stress response.
- By screening for the ABA response of a wide array of ERAD mutants, we isolated a gain-of-function mutant, *doa10a-1*, which conferred ABA hypersensitivity to seedlings.
- Genetic and biochemical assays showed that DOA10A is a functional E3 ubiquitin ligase which, by acting in concert with specific E2 enzymes, mediates mono-ubiquitination of the ABA receptor, followed by their relocalization to the plasma membrane. This in turn leads to enhanced ABA perception.
- In summary, we report here the identification of a novel RING-type E3 ligase, DOA10A, which regulates ABA perception by affecting the localization and the activity of ABA receptors through their mono-ubiquitination.

Introduction

The phytohormone abscisic acid (ABA) is a key mediator of the plant response to environmental stresses ranging from drought, to cold, to virus infections (Zhang *et al.*, 2007; Ding *et al.*, 2015; Zhu, 2016) and regulates many aspects of plant growth and development, including seed germination, cotyledon greening and seedling growth (Finkelstein *et al.*, 2002; Delmas *et al.*, 2013). Despite its complexity, the ABA signaling pathway can be reconstructed with four core components *in vivo*: pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/regulatory components of ABA receptors (RCAR) family, protein phosphatases type 2Cs (PP2Cs), sucrose nonfermenting 1-related protein kinases (SnRK2s) and the transcription factor ABA-responsive element binding factors (ABFs/AREBs) (Furihata *et al.*, 2006; Fujii *et al.*, 2007, 2009; Ma *et al.*, 2009; Park *et al.*, 2009; Umezawa *et al.*, 2009). Under low ABA levels, PP2Cs interacts with SnRK2s, thus inhibiting their kinase activity and blocking the ABA signaling cascade. Under stress conditions, ABA levels increase, allowing ABA binding to its cognate receptors PYR1/PYLs/RCARs, and enabling them to sequester PP2Cs. This releases SnRK2-mediated induction of the downstream ABFs/AREBs transcription factors and triggers cellular responses such as the control of the metabolic rate and ion channels opening (Nishimura *et al.*, 2009; Yin *et al.*, 2009; Cutler *et al.*, 2010).

Considerable effort has been made to identify the regulators of the ABA signaling cascade. Among them, the ubiquitin (Ub)/26S proteasome system (UPS) has been found to be involved in ABA signaling at several levels (Yu *et al.*, 2016a, 2016b). For example, the E3 ubiquitin ligase keep on going (KEG) and cullin-RING ligase 4 (CRL4) mediate ubiquitination and subsequent degradation of ABA-insensitive 5 (ABI5), a bZIP transcription factor belonging to the ABF family in ABA signaling (Stone *et al.*, 2006; Lee *et al.*, 2010; Seo *et al.*, 2014). When ABI1, a member of the PP2Cs in ABA signaling, interacts with ABA receptors, it is ubiquitinated by the E3 ligases plant U-box 12 (PUB12) and PUB13

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(Kong *et al.*, 2015). In the last few years, several reports have shown that the E3 ligase RING finger of seed longevity 1 (RSL1), substrate adaptor de-etiolated 1 (DET1)-, damaged DNA-binding protein 1 (DDB1)-associated 1 (DDA1) from the CRL complex and rice tiller enhancer (TE), an activator of anaphase-promoting complex/cyclosome (APC/C), can all mediate ubiquitination and degradation of the ABA receptors (Bueso *et al.*, 2014; Irigoyen *et al.*, 2014; Lin *et al.*, 2015). Besides its role in protein degradation, ubiquitination also regulates protein interactions, activity and localization depending on the type of ubiquitination (mono- or poly-) and the linkage type of the ubiquitin chains (Komander & Rape, 2012). Among them, mono-ubiquitination and K63-linked ubiquitination often affect endomembrane trafficking and subcellular localization of the target proteins (Clague & Urbe, 2010; Yu & Xie, 2017).

Ubiquitination is essential for the proper function of the endoplasmic reticulum-associated degradation system (ERAD), which targets and eliminates misfolded proteins to maintain protein cellular homeostasis (Liu & Howell, 2010; Smith *et al.*, 2011). Stressful environmental conditions, such as water scarcity, abnormally low or high temperature, or high salt concentrations, affect the protein folding process, and induce accumulation of misfolded proteins in the ER, a condition known as ER-stress (Hoseki *et al.*, 2010). These overaccumulated proteins are ubiquitinated by ERAD-specific E3 ubiquitin ligases, and then degraded through the proteasome and/or the lysosome/vacuole to alleviate ER stress. We have previously reported that ERAD participates in plant abiotic stress response (Liu *et al.*, 2011; Cui *et al.*, 2012; Chen *et al.*, 2021).

Taken together, these observations all point to the possibility that ERAD and the ABA pathway might interact to properly regulate the plant response to abiotic stress. We thus decided to investigate the relationship between ABA and ERAD by performing a screen for altered ABA responses among *Arabidopsis thaliana* mutants in genes homologous to selected yeast ERAD components. We report here the identification of a gain-of-function mutant, which we named *doa10a-1*, which is hypersensitive to ABA. We show that *Arabidopsis* DOA10A (homolog of the yeast ERAD-associated E3 ligase DOA10) encodes an E3 ubiquitin ligase which mono-ubiquitinates PYR/PYL family members with the assistance of the E2 conjugase enzyme UBC3 on specific amino acid residues. This triggers relocalization of mono-ubiquitinated receptors, which move to the plasma membrane through the endomembrane system, and leads to enhanced ABA sensitivity.

Materials and Methods

Plant materials and growth conditions

The *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used as control. *doa10a-1* (*cer9-2*), *ubc32*, *hrd3a*, *pyr1/pyl1/2/4*, *abi1-1* (Col-0), *snrk2.2/2.3* and *abi5-7* mutants were reported previously (Park *et al.*, 2009; Liu *et al.*, 2011; Cui *et al.*, 2012; Lü *et al.*, 2012). Mutant seeds were ordered from the *Arabidopsis* Biological Resource Center (ABRC; The Ohio State University, Columbus, OH, USA). Seeds were sterilized with 10% bleach and washed four times with sterilized H_2O, then plated on 1/2 MS medium with or without ABA as needed, at the concentration indicated in the text. Seeds were cultured at 22°C under a 16 h : 8 h, light : dark photoperiod after stratification in darkness for 3 d at 4°C. After 14 d, seedlings were transferred into soil and grew in glasshouse of 22°C and 70% RH under a 16 h : 8 h, 22°C, light : dark photoperiod.

Vectors construction and generation of transgenic plants

Vectors used for bimolecular fluorescence complementation (BiFC) assay, luciferase complementation imaging (LCI) assay and the reconstitution of the ABA signaling were reported previously (Fujii *et al.*, 2009). The marker proteins mcherry-HDEL, Man49-mcherry, mRFP-SYP61 and mRFP-VSR2 were used in previous study (Tian *et al.*, 2015). Full-length coding sequence (CDS) of *DOA10A* (At4g34100) was cloned into pCAMBIA1300-cLUC between *Kpn* I and *Sal* I to construct DOA10A-cLuc, used in LCI assay, generation of transgenic plants and transient expression system. The nucleotide sequence coding the first N-terminal 150 amino acids of DOA10A was cloned into pCAMBIA1300-cLUC between *Kpn* I and *Sal* I to construct $DOA10A^{1-150}$-cLuc, used in LCI assay, generation of transgenic plants and transient expression system. The nucleotide sequence coding N-terminal 150 amino acids of DOA10A was cloned into pGEX4T between *Eco*R I and *Sal* I for bio-layer interferometry (BLI) assay and *in vitro* ubiquitination assay. Full-length CDS of *DOA10A* was cloned into pUC-SPYCE between *Sal* I and *Kpn* I, resulting in cYFP-DOA10A for BiFC. Full-length CDS of *PYR1* (At4g17870) was cloned into pUC-SPYNE between *Sal* I and *Kpn* I, resulting in PYR1-nYFP for BiFC. Full-length CDS of *PYR1* was cloned into pCAMBIA1300-nLUC between *Kpn* I and *Sal* I for LCI assay. Full-length CDS of *PYR1* was cloned into pET28a between *Eco*R I and *Sal* I for protein expression in bacteria. *PYR1* was cloned into pSuper-Flag between *Xho* I and *Spe* I for ubiquitination assay. *PYR1* was cloned into pGFP2 between *Xho* I and *Kpn* I for protein transient expression in tobacco. Full-length coding region of *PYR1* and *Ub* (At4g02890) were fused and cloned into pGFP2 between *Sal* I and *Sac* I with a GGGSGGG linker as described (Terrell *et al.*, 1998) to construct GFP-PYR1-Ub for transient transfection. *PYR1* and *PYR1-Ub* were cloned into pCAMBIA1300-GFP.3 between *Bam*H I and *Sac* I for overexpression in *Arabidopsis*. *pYAO:hSpCas9-DOA10A-sgRNA* was constructed using ATGGACAACGAATCGGCCG as the sgRNA. All primers used were listed in Supporting Information Table S1.

All transgenic plants were generated using vacuum infiltration of *Arabidopsis* inflorescences with the *Agrobacterium tumefaciens* strain GV3101 (Zhang *et al.*, 2006). T_2 seeds were selected using 20 mg ml^{-1} hygromycin to obtain resistant and homozygous T_3 seeds. Two independent homozygous and single insertion lines were used for all the experiments.

In-gel kinase assay

Crude extracts were prepared from 10-d-old seedlings treated in 1/2 MS liquid medium with or without 50 μM ABA for 30 min

and separated by SDS-PAGE gel containing 0.1 mg ml^{-1} MBP substrate. The in-gel kinase assay was conducted as reported previously (Fujii *et al.*, 2007).

RT-PCR and qRT-PCR

Total RNA was prepared from 10-d-old seedlings treated in 1/2 MS liquid medium with or without 50 μM ABA for the times indicated in the figures. 2 μg total RNA was used for reverse transcription. All primers used were listed in Table S1. The CFX96 real-time system (Bio-Rad) was used for quantitative reverse transcription polymerase chain reaction.

LCI assay

LCI assay was performed as reported previously (Chen *et al.*, 2008). The CDS of *DOA10A* and *PYR1/PYLs* was fused to the LUC coding sequence at the C-terminus or at the N-terminus, respectively. Different pairwise combinations of equal amounts of *Agrobacterium* strains (at the concentration of OD_{600} = 1.0 (p19, OD_{600} = 0.6)) carrying the different constructs were mixed and injected into one of four different sectors of a *N. benthamiana* leaf. The luciferase substrate was smeared on the leaf 3 d post infection and the chemiluminescent image was captured by a low-light cooled charge-coupled device imaging apparatus (NightOWL II LB983).

BiFC assay

BiFC was performed as reported previously (Bai *et al.*, 2007). The full-length CDS of *DOA10A* and *PYR1* was fused to the C-terminus and N-terminus portions of YFP to produce cYFP-DOA10A and nYFP-PYR1, respectively. cYFP-CPK8 and ABI5-nYFP were used as the negative controls. Equal amounts of *Agrobacterium* GV3101 strains carrying individual constructs at the concentration of OD_{600} = 1.0 (p19, OD_{600} = 0.6) were mixed and injected in *N. benthamiana* leaves. YFP fluorescence was observed 3 d post infection using a laser confocal microscope (Leica).

BLI assay

The binding affinity assay between $DOA10A^{1–150}$ and full-length *PYR1* was performed using a BLI technology (OctetRED system, ForteBio). GST-$DOA10A^{1–150}$ and His-PYR1 fusion proteins were expressed in *Escherichia coli* and purified. 4 μg GST-$DOA10A^{1–150}$ or GST (control) was bound on the sensors, and His-PYR1 was diluted into different concentrations (0, 2.5, 5, 10, 20, 40 μM) with 50 mM Tris–HCl (pH 7.4). The association/dissociation of DOA10A–PYR1 was monitored for 10 min at 25°C.

In vitro ubiquitination assay

The *in vitro* ubiquitination assay was performed as reported previously (Zhao *et al.*, 2013). Wild-type (WT) and mutated Flag-PYR1-fusion proteins were expressed and immunoprecipitated from *N. benthamiana* leaves, while other proteins used were expressed and purified in *E. coli*. The proteins used in the reaction mixture were His-E1 (*Arabidopsis* UBA2, 50 ng), His-E2 (100 ng), GST-E3 ($DOA10A^{1–150}$, 200 ng), Ub (*Arabidopsis* ubiquitin 14, 500 ng), PYR1 (50 ng) and deubiquitinating enzyme (EBV-DUB from Epstein–Barr virus) (Whitehurst *et al.*, 2009).

Immunoprecipitation

Crude proteins were extracted using native buffer 50 mM Tris-MES pH 8.0, 0.5 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA, 5 mM DTT, 1 mg ml^{-1} protease inhibitor cocktail (Complete Mini tablets; Roche C756V54) from 10-d-old seedlings or *N. benthamiana* leaves. Tissues were ground using liquid nitrogen and resuspended in an equal volume of extraction buffer. After centrifugation at 16 000 ***g*** at 4°C for 10 min, 1 ml supernatant was subjected to immunoprecipitation using Anti-Flag M2 beads as described (M8823; Sigma-Aldrich). The PYR1-Flag fusion protein was eluted with the Flag peptide (F3290; Sigma-Aldrich), and used for *in vitro* ubiquitination assay.

Protoplast preparation and transient gene expression assays

Protoplast preparation and transient gene expression assays were performed as reported previously (Yoo *et al.*, 2007). All plasmids were purified using QIAGEN Plasmid Maxi Kit, and 10 μg plasmid was used for protoplast transfection. Leaves were obtained from plants at the vegetative stage, cut into strips and incubated in enzyme solution. Protoplast release from tissues was monitored with an optic microscope. When most of the protoplasts were released, they were collected, counted and 2×10^4 protoplasts were used for each transfection. Incubation was carried out different time as indicated in the text.

The ABA signal transduction reconstitution assay was performed as reported previously (Hou *et al.*, 2016). Protoplasts were collected after 4-h incubation with or without 5 μM ABA under light and resuspended in 60 μl lysis buffer. For LUC assays, 30 μl of protoplast lysate was used (P1041; Promega). For GUS assays, 2 μl of protoplast lysate was incubated with 10 μl 2 mM 4-methylumbelliferyl β-D-glucuronide (MUG) substrate for 30 min at 37°C, and the reaction was stopped by the addition of 200 μl 0.2 M Na_2CO_3. The fluorescence was detected using a 355 nm excitation filter and a 460 nm emission filter.

Results

A gain-of-function mutation of *DOA10A* enhances ABA sensitivity

To investigate the relationship between the ERAD and ABA pathways, we first identified the *Arabidopsis* putative counterparts of yeast genes coding for selected ERAD components involved in

the ubiquitination pathway (Table S2). We next monitored the capability of their corresponding available mutants to establish a proper seedling upon addition of exogenous ABA (Fig. S1). While all lines grew similarly in normal medium, several were affected in their growth capability upon the addition of exogenous ABA (Fig. S1). Among those, *doa10a-1*, a mutant in the *Arabidopsis DOA10A* orthologous gene (Table S2) was particularly sensitive to the ABA-mediated inhibition of seedling establishment compared with the WT (Col-0) and is thus further described in this manuscript (Figs 1a, S1, S2a). Interestingly, *Arabidopsis DOA10A* has been already described as *ECERIFERUM9* (*CER9*) (Koornneef *et al.*, 1989) and shown to be involved in wax and cutin synthesis, as the leaves of its mutant alleles *cer9-1* and *cer9-2* have a thicker cuticle membrane, leading to lower transpiration rates and drought tolerance (Koornneef *et al.*, 1989; Lü *et al.*, 2012). Sequence analysis further confirmed that the *doa10a-1* mutation corresponds to the *cer9-2* mutation. Unexpectedly, *DOA10A* overexpression was only able to partially rescue ABA hypersensitivity of *doa10a-1* seedlings (Figs 1a,b, S2b), which is consistent with the previous study (Etherington *et al.*, 2023). And seedlings heterozygous for the *doa10a-1* mutation were hypersensitive to ABA (Fig. 1c,d). Further, a CRISPR-Cas9-mediated knockout of *DOA10A* in both Col-0 and *doa10a-1* was generated, which had a base insertion or a base deletion in the first exon, resulting in the early translation termination with 27 aa and 25 aa separately (Fig. S2c–e). The CRISPR-Cas9 gene editing of *DOA10A* rescued the ABA hypersensitivity of *doa10a-1* (Fig. 1e,f). In addition, WT seedlings overexpressing *DOA10A* also showed ABA hypersensitivity (Figs 1g,h, S2f). Based on this evidence, we concluded that the *doa10a-1* mutation had a dominant effect. Since the *doa10a-1* mutant has a T-DNA inserted in the fifth exon of the *DOA10A* gene (Fig. S2a; Lü *et al.*, 2012), we speculated that the dominant character of the *doa10a-1* mutation could be due to the production of a functional truncated protein. Indeed, Lü *et al.* (2012) reported the existence of a *DOA10A* truncated transcript downstream of the T-DNA insertion site in *cer9-2* (Lü *et al.*, 2012). Based on the sequence and site of insertion of T-DNA, the truncated protein, which we called DOA10A-1, should contain only the first 714 amino acids of the full-length protein, which include the N-terminal RING domain, but lack the C-terminal 7 transmembrane domains (Fig. S2c). This could have severe consequences for the E3 ligase activity of the mutated protein DOA10A-1. Indeed, overexpression of the first N-terminal 150 amino acids of DOA10A ($DOA10A^{1–150}$), which include the RING domain, but lack the transmembrane region, enhanced the ABA sensitivity of WT (Col-0) seedlings (Fig. S3). Taken together, these results suggested that the ABA hypersensitivity of *doa10a-1* is caused by a gain-of-function effect of the *doa10-1* mutation.

A gain-of-function mutation of *DOA10A* activates ABA signaling in ABA receptor mutant

To investigate how *DOA10A* participates in the ABA signaling pathway, we generated different double mutant combinations of the *doa10a-1* mutant with mutants of core components of the ABA signaling pathway, and monitored their response to exogenous ABA. As shown in Fig. S4a,b, the *abi1-1* (Wu *et al.*, 2003), *snrk2.2/2.3* (Fujii & Zhu, 2019) and *abi5-7* (Nambara *et al.*, 2002) mutations were all able to completely rescue the ABA hypersensitivity of the *doa10a-1* mutant, suggesting that *DOA10A* plays a role upstream of these components in the well-recognized PYR/PYL mediated ABA signal pathway. Interestingly, the *doa10a-1* mutation was able to enhance the ABA sensitivity of the *pyr1 pyl1/2/4* quadruple mutant (Figs 2a,b, S4). To confirm whether this was due to a restoration of the ABA signaling cascade in the *doa10a pyr1 pyl1/2/4* quintuple mutant, we assessed both the kinase activity of SnRK2.6 and the transcription level of ABA-responsive genes including *RD29A*, *RD29B*, *RD22* and *RAB18*. As shown in Figs 2c,d, S4e, the SnRK2.6 kinase activity and *RD29A*, *RD29B* and *RD22* transcription were inhibited in the *pyr1 pyl1/2/4* quadruple mutant when compared with the WT, but they were restored in the *doa10a-1 pyr1 pyl1/2/4* quintuple mutant. These results suggest that the *doa10a-1* gain-of-function mutation could reactivate ABA signaling in the *pyr1 pyl1/2/4* quadruple mutant. Since the ABA receptor family includes 14 members (Ma *et al.*, 2009; Park *et al.*, 2009), only four of which are mutated in *pyr1 pyl1/2/4*, we speculated that the *doa10a-1* gain-of-function mutation might enhance the activity of the remaining ABA receptor family members, thus leading to a reactivation of the ABA signaling.

DOA10A mediates ubiquitination of ABA receptor

DOA10A encodes a putative E3 ligase and could thus mediate ubiquitination of specific substrates. This, together with the effect of the *doa10a-1* mutation on the ABA sensitivity of *pyr1 pyl1/2/4* seedlings, prompted us to verify, first, whether DOA10A is a functional E3 ligase and, second, if it mediates ubiquitination of members of the ABA receptors family. As the full-length DOA10A is a large multi-transmembrane protein, which is difficult to express and manipulate *in vitro*, we decided to examine the self-ubiquitination of the truncated $DOA10A^{1–150}$ protein in an *in vitro* assay. Fig. 3a shows that GST-$DOA10A^{1–150}$ was able to mediate its self-ubiquitination – as evident by the formation of a ladder of higher MW products – only when paired with the E2 conjugase UBC3. This demonstrates that, similar with its yeast orthologue, DOA10A is a functional E3 ubiquitin ligase.

Next, in order to explore whether ABA receptors could be DO10A substrates, their possible interaction with DO10A was assayed using a BiFC assay. As shown in Fig. 3b, we were able to detect fluorescence when cYFP-DOA10A and PYR1-nYFP, but not their empty vector controls, were co-injected in tobacco leaves, indicating an interaction between the two proteins. To assess whether DOA10A also interacts with other members of the ABA receptor family, we used a luciferase complementation imaging (LCI) assay. Indeed, a full-length DOA10A was able to interact with all ABA receptor family members, from PYR1 to PYL1-13 (Fig. S5a). In addition, LCI assay showed the truncated $DOA10A^{1–150}$ protein also interacts with the PYR1 (Fig. S5b).

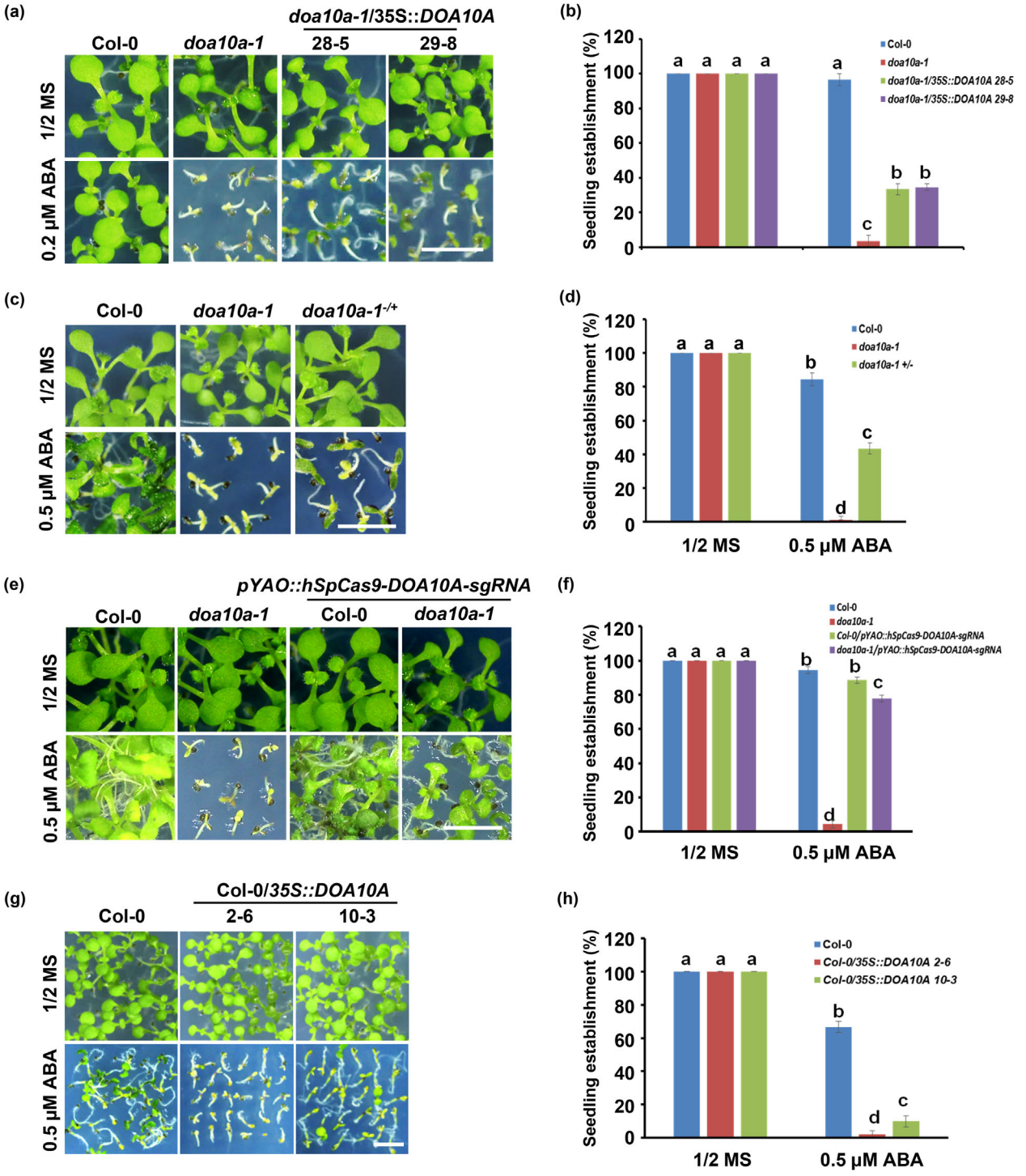

Fig. 1 The *doa10a-1* gain-of-function mutation enhances abscisic acid (ABA) sensitivity. (a, b) The *doa10a-1* mutation is hypersensitive to ABA-mediated inhibition of seedling establishment; overexpression of *DOA10A* in the *doa10a-1* mutant (*doa10a-1/35S::DOA10A*) does not rescue its sensitivity. Seedlings were photographed (a) and the seedling establishment rates were determined (b) at 8 d post sowing. (c, d) Both homozygous (*doa10a-1*) and heterozygous (*doa10a-1*$^{-/+}$) seedlings are more sensitive to ABA-mediated inhibition of seedling establishment than wild-type (WT) control (Col-0). Seedlings were photographed (c) and the seedling establishment rates were determined (d) at 12 d post sowing. (e, f) Knocking out *DOA10A* reduces the sensitivity of the *doa10a-1* mutant to ABA. *doa10a-1* seedlings harboring a knock-out deletion in the *DOA10A* gene (*pYAO*::hSpCas9-*DOA10A*-sgRNA) were photographed (e) and the seedling establishment rates were determined (f) at 12 d post sowing. (g, h) Overexpression of *DOA10A* (Col-0/35S-DOA10A) enhances the ABA sensitivity of WT seedlings. Seedlings were photographed (g) and the seedling establishment rates were determined (h) at 8 d post sowing. Different numbers indicate independent lines. In (b, d, f and h), error bars represent the SD (triplicate measurements; $n = 25$). Different letters indicate significantly different groups ($P < 0.05$, ANOVA, Tukey's HSD). All seeds were sown on 1/2 MS medium, with or without ABA at the concentration indicated in the figure. Bar, 0.5 cm.

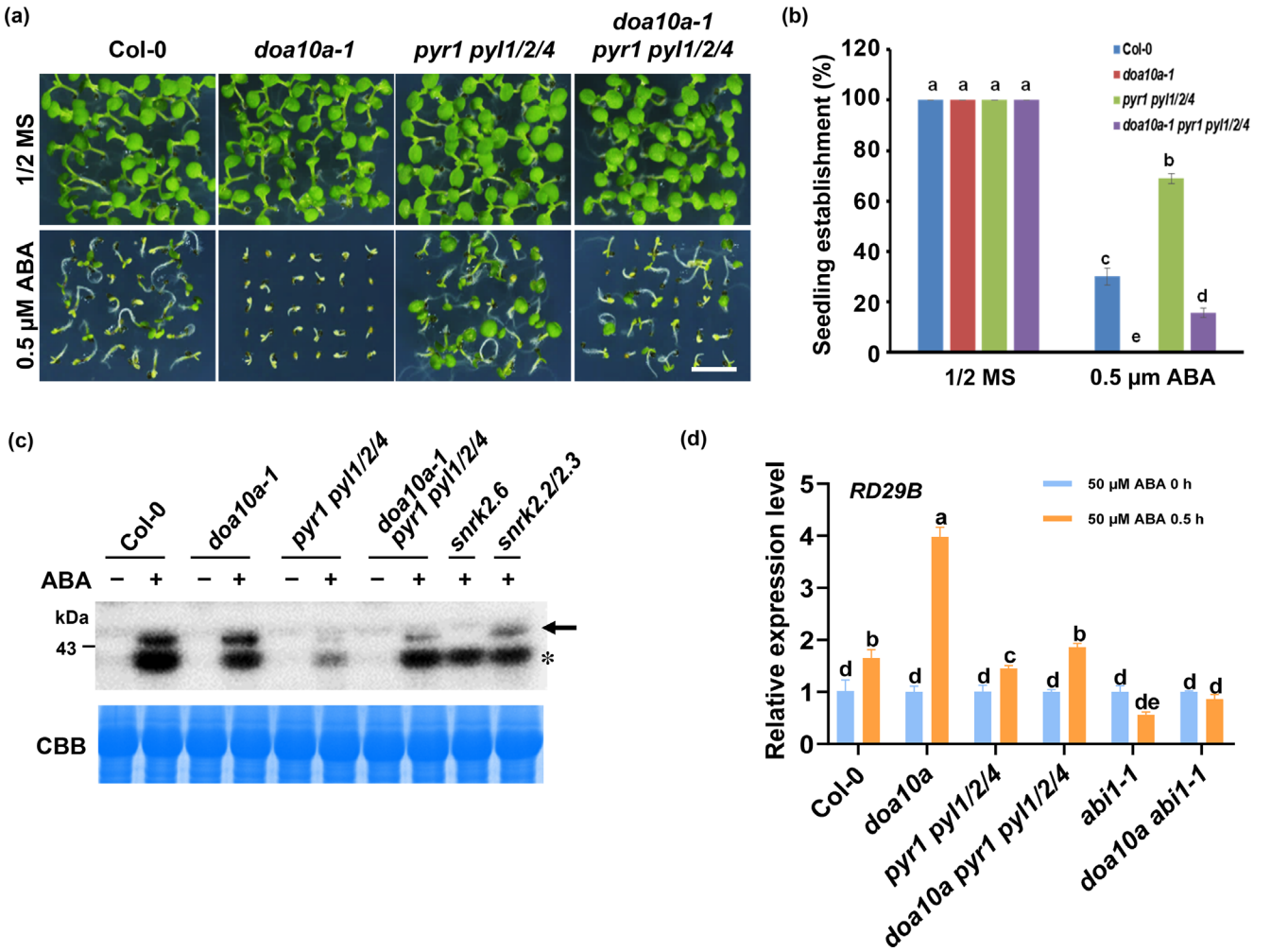

Fig. 2 The gain-of-function mutation *doa10a-1* activates abscisic acid (ABA) signaling in a quadruple ABA receptor mutant. (a) The *doa10a-1* mutation enhances the sensitivity of the quadruple *pyr1/pyl1/2/4* mutant to ABA-mediated inhibition of seedling establishment. Seeds were sown on 1/2 MS medium with or without 0.5 μM ABA. At 8 d post sowing, seedlings were photographed. Bar, 0.5 cm. (b) Quantification of the seedling establishment rates in (a). Error bar represents the SD (triplicate measurements; $n = 30$). Different letters indicate significantly different groups ($P < 0.05$, ANOVA, Tukey's HSD). (c) The *doa10a-1* mutation enhances the ABA-mediated kinase activity of SnRK2.6/OST1 in the *pyr1 pyl1/2/4* mutant. Total proteins, for in-gel kinase assay, were extracted from 10-d-old seedlings treated with or without 50 μM ABA for 30 min. Upper panel, autoradiography of kinase activity. The phosphorylated signal of SnRK2.6/OST1 substrate GST1-ΔABF2 was indicated by arrow. Asterisks indicate nonspecific bands. Lower panel, coomassie blue staining of the RUBISCO large subunit as a loading control. (d) The *doa10a-1* mutation enhances the ABA-induced expression of *RD29B* in the *pyr1 pyl1/2/4* mutant. For quantitative polymerase chain reaction assay, RNA was extracted from 10-d-old seedlings treated with or without 50 μM ABA for 30 min. *ACTIN2* was used as the internal control. Error bars represent the SD (triplicate measurements). Different letters indicate significantly different groups ($P < 0.05$, ANOVA, Tukey's HSD).

This was also confirmed by a BLI (BLI) assay, which indicated that DOA10A$^{1–150}$ protein interacts directly with PYR1 (Fig. 3c). Thus, the E3 ubiquitin ligase DOA10A interacts with ABA receptors both *in vivo* and *in vitro*, most likely through its N-terminal region.

To verify whether DOA10A mediated ubiquitination of the ABA receptors, we performed an *in vitro* substrate ubiquitination assay using GST-DOA10A$^{1–150}$, UBC3 and PYR1-Flag and examined PYR1-Flag ubiquitination by its mobility shift. As shown in Fig. 3d, the presence of two higher MW bands above the unmodified form of PYR1 indicated that, when paired with UBC3, DOA10A was able to ubiquitinate PYR1. PYR1 ubiquitination also occurred when UBC1 or UBC2, which belong to the same E2 subfamily with UBC3 (Table S3), were used instead of UBC3. The addition of a deubiquitinating enzyme (DUB), which cleaves ubiquitin from ubiquitinated proteins (Ernst *et al.*, 2011), effectively reduced the amount of ubiquitinated forms of PYR1, indicating that PYR1 was indeed modified by ubiquitin via the DOA10A-UBC3 pair (Fig. 3d). We then asked whether DOA10A was able to ubiquitinate other ABA receptors. Indeed, swapping PYR1 with each other members of the ABA receptor family in both the LCI and the *in vitro* substrate ubiquitination assays indicated that all ABA receptors could be ubiquitinated by the DOA10A-UBC3 pair (Fig. S5c). Taken together, these results demonstrated that ABA receptors are ubiquitination substrates of DOA10A.

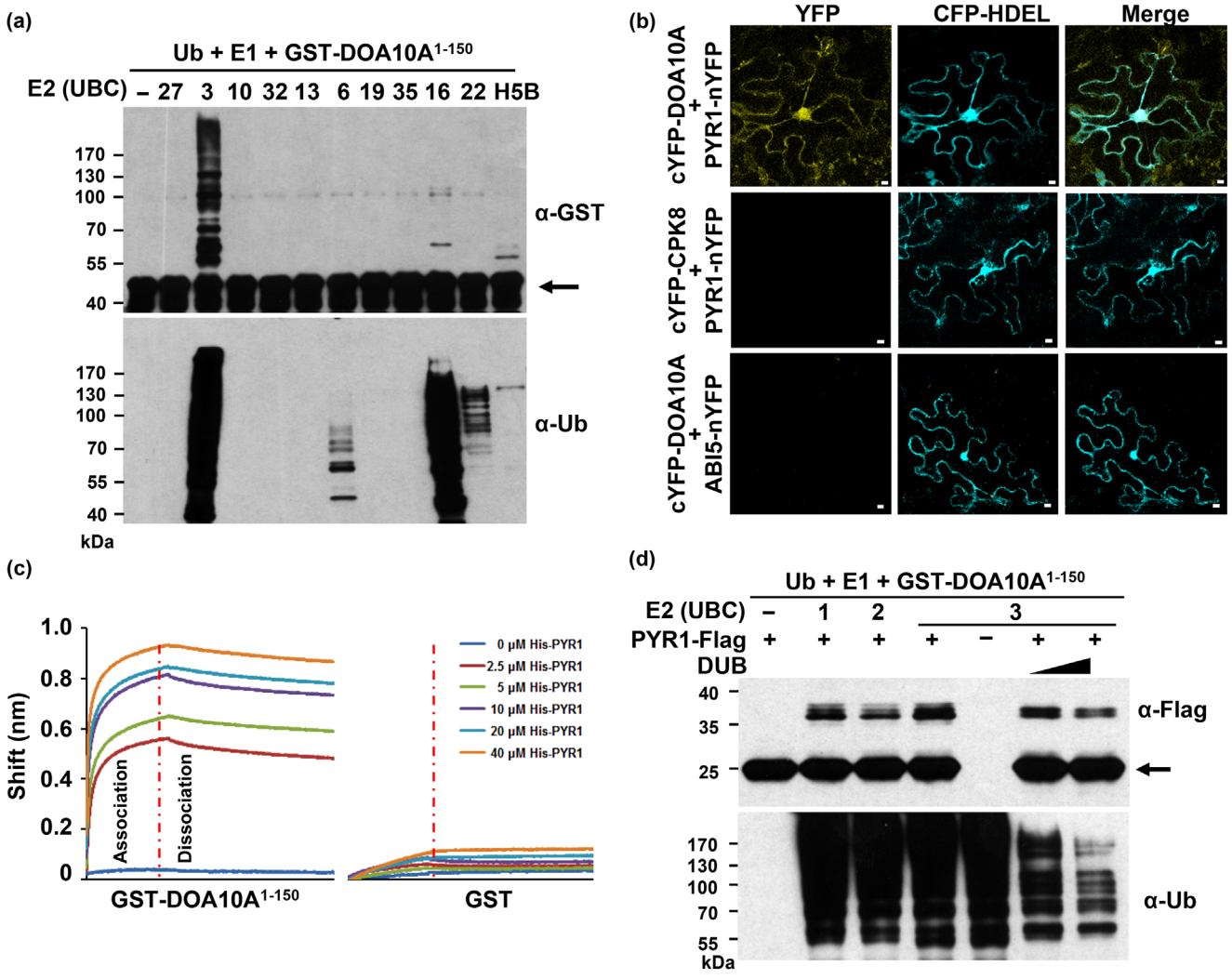

Fig. 3 DOA10A mediates ubiquitination of the abscisic acid (ABA) receptor PYR1. (a) DOA10A has ubiquitin ligase activity when coupled with the E2 conjugase UBC3 *in vitro*. Upper panel, GST-DOA10A$^{1–150}$ was mixed with the reagents indicated in the figure and listed in Supporting Information Table S3. The samples were subjected to immunoblot analysis with anti-GST antibodies. Multiple E2s (UBCs) were assayed in this reaction including UBC27, UBC3, UBC10, UBC32, UBC13, UBC6, UBC19, UBC35, UBC16, UBC22 and human E2 UbcH5B. The arrow indicates GST-DOA10A$^{1–150}$, while higher MW forms correspond to ubiquitinated forms of GST-DOA10A$^{1–150}$. Lower panel, the same samples as the upper panel were subjected to an immunoblot with anti-ubiquitin antibodies. (b) DOA10A interacts with PYR1 *in vivo*. Bimolecular fluorescence complementation assay between cYFP-DOA10A and PYR1-nYFP. Reconstitution of YFP fluorescence denotes interaction. CFP-HDEL (endoplasmic reticulum marker protein), and cYFP-CPK8 and ABI5-nYFP were used as controls. Bar, 10 μm. (c) DOA1 interacts with PYR1 *in vitro*. Bio-layer interferometry (BLI) assay traces the binding affinity of PYR1 to DOA10A$^{1–150}$, showing the binding kinetics of different concentration of PYR1-Flag with GST-DOA10A$^{1–150}$ immobilized on the BLI sensor. The GST protein was used as control. (d) DOA10A ubiquitinates PYR1 directly *in vitro*. Upper panel, GST-DOA10A$^{1–150}$ was mixed with the reagents indicated in the figure. The samples were subjected to immunoblot analysis with anti-Flag (upper) or anti-ubiquitin (lower) antibodies. The numbers indicate the different E2 (UBC) enzyme added to the reaction. The arrow indicates PYR1-Flag, while higher MW bands indicate ubiquitinated forms of PYR1-Flag. DUB, deubiquitinating enzyme. The triangle indicates increasing DUB concentrations.

DOA10A-mediated PYR1 mono-ubiquitination alters its subcellular localization

The final outcome of the ubiquitination cascade is usually the formation of a covalent bond between ubiquitin and one of more lysine residues on the substrate protein. The presence of two higher MW bands above the unmodified form of PYR1 in Fig. 3c suggests that PYR1 could be mono-ubiquitinated by DOA10A at two distinct amino acid residues. To identify the DOA10A-mediated ubiquitination sites of PYR1, each one of PYR1 lysines (K) was mutated to an arginine (R). The obtained PYR1 K to R variants were used in an *in vitro* substrate ubiquitination assay with the DOA10A-UBC3 pair. As shown in Fig. 4a, only one ubiquitinated band was observed when using the PYR1 (K14R)-Flag and PYR1 (K63R)-Flag variants as substrates, implying that PYR1 was mono-ubiquitinated on its K14 and K63 residues. Indeed, both ubiquitinated bands were diminished, when a double-mutant form of PYR1-Flag (K14/63R) was used (Fig. 4b). Thus, we concluded that DOA10A mediates the mono-ubiquitination of PYR1 on its K14 and K63 residues.

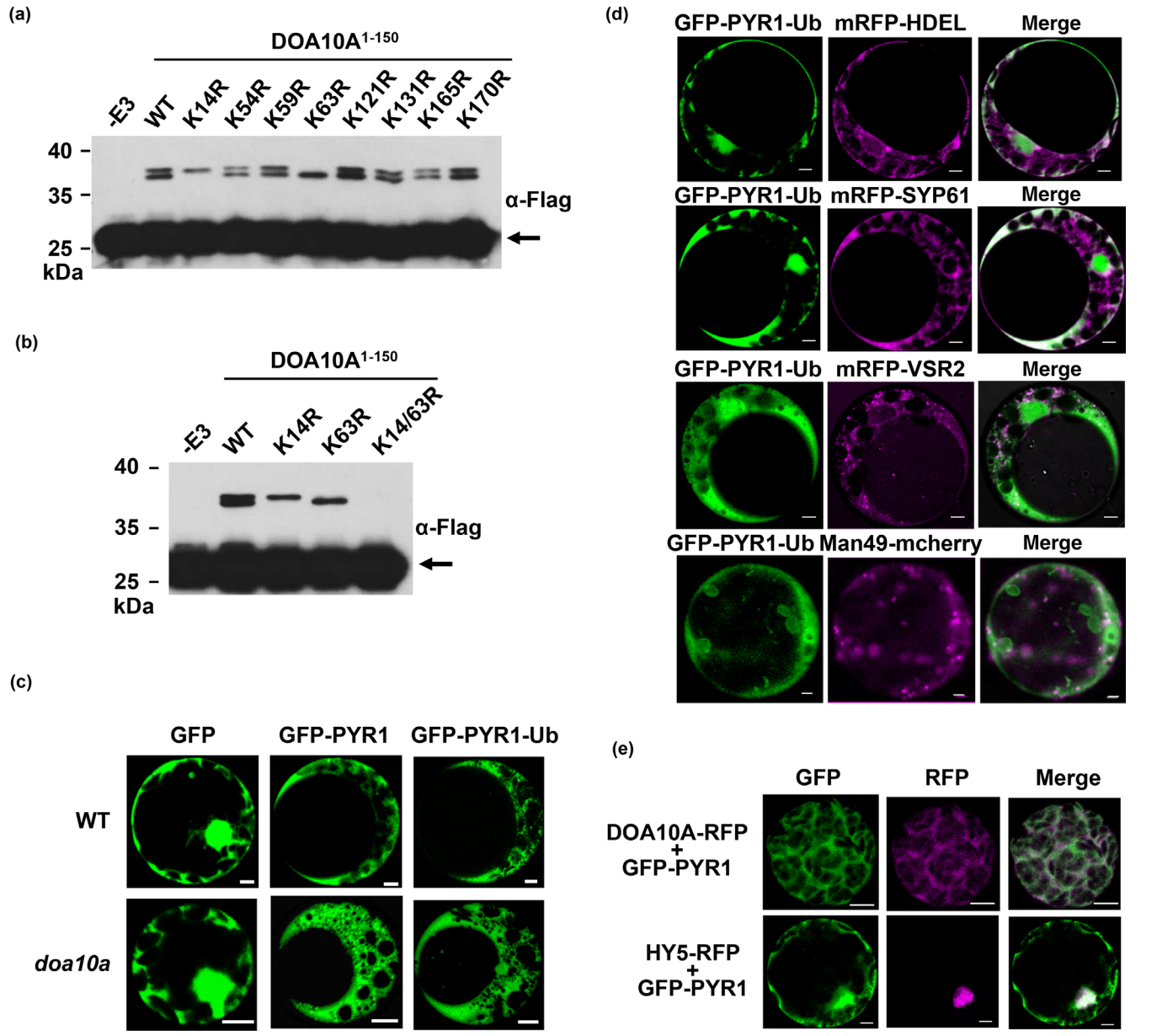

Fig. 4 DOA10A-mediated mono-ubiquitination of PYR1 affects its subcellular localization. (a, b) DOA10A mediates mono-ubiquitination of PYR1-Flag at K14 and K63. Each lysine (K) of PYR1-Flag was mutated to arginine (R) and used in an *in vitro* ubiquitination assay with GST-DOA10A$^{1–150}$, E1, UBC3 and the FLAG fusion of different PYR1 variants indicated in the figure. E3 indicates addition only of GST. WT, wild-type PYR1. The arrows indicate unmodified PYR1-Flag, and higher MW bands corresponds to ubiquitinated forms ofPYR1-Flag. K14/63R denotes the double variant harboring K14R and K63R. (c) Fusion of an ubiquitin at the C-terminus of PYR1 affects its subcellular localization. Constructs encoding GFP, GFP-PYR1 or GFP-PYR1-Ub were transfected into Col-0 and *doa10a Arabidopsis* protoplasts, respectively. GFP fluorescence was observed after 16-h transfection. Bar, 5 μm. (d) Colocalization of PYR1-Ub and subcellular compartment markers. A construct encoding GFP-PYR1-Ub was cotransfected with the markers indicated in the figure into *Arabidopsis* protoplasts, respectively. GFP and RFP fluorescence were observed after 16-h transfection. mRFP-HDEL, endoplasmic reticulum marker. mRFP-SYP61, TGN/EE marker. mRFP-VSR2, PVC marker. Man49-mcherry, *cis*-Golgi marker. Bar, 5 μm. (e) Addition of DOA10A affects PYR1 subcellular localization. A construct coding for GFP-PYR1 was co-transfected with DOA10A-RFP or HY5-RFP into *Arabidopsis* protoplasts. GFP and RFP fluorescence was observed after 16-h transfection. HY5 localizes to the nucleus, and was used as a transfection control. Bar, 5 μm.

It has been shown that ABA receptors can be ubiquitinated and that this affects their subcellular localization (Bueso *et al.*, 2014; Yu *et al.*, 2016a, 2016b). To investigate whether mono-ubiquitination could affect PYR1 subcellular localization, we fused an ubiquitin to the C-terminus of a GFP-PYR1 fusion (GFP-PYR1-Ub) to mimic mono-ubiquitination, using a previously described strategy (Terrell *et al.*, 1998). Constructs coding for GFP-PYR1 (control) and GFP-PYR1-Ub were transiently transfected into Col-0 and *doa10a Arabidopsis* protoplasts and the subcellular localization of the two fusion proteins was monitored. Compared with the location of PYR1 in Col-0, the subcellular localization of PYR1 in doa10a was significantly

changed with more dot localization, which was similar to the subcellular localization of PYR1-Ub. The results are shown in Fig. 4d and indicate that, while GFP-PYR1 localizes to the endosome compartment (Belda-Palazon *et al.*, 2016; Yu *et al.*, 2016a, 2016b), the GFP-PYR1-Ub mono-ubiquitination mimic formed multiple larger or/and more punctations (Fig. 4c). However, PYR1 has weak colocalization with endosome compartment (Fig. S6). A further analysis using different intracellular compartment markers confirmed that GFP-PYR1-Ub also localizes to the endomembrane system (Fig. 4d). Interestingly, when co-expressed with the E3 ubiquitin ligase DOA10A, GFP-PYR1-Ub and GFP-PYR1 subcellular localization profiles became very similar. This did not occur when a control protein (HY5-RFP) was co-expressed with GFP-PYR1 (Fig. 4e). In conclusion, these results suggest that DOA10A-mediated mono-ubiquitination affects the subcellular relocalization of PYR1.

Constitutive expression of a PYR1 mono-ubiquitination mimic enhances ABA perception

Since mono-ubiquitination of PYR1 affects its subcellular localization, this could in turn regulate its cellular function(s). To assess this hypothesis, we constructed transgenic lines overexpressing either *GFP-PYR1* or *GFP-PYR1-Ub*, respectively. We noticed that, although the protein level of GFP-PYR1-Ub in different transgenic lines was often lower than the levels of GFP-PYR1 (Fig. S7), the *GFP-PYR1-Ub* transgenic lines were more sensitive to ABA than the lines expressing *GFP-PYR1* alone (Fig. 5a,b). Indeed, the expression level of *RD29B*, a transcription factor downstream of the ABA signaling pathway, was higher in lines overexpressing *GFP-PYR1-Ub* than in those overexpressing *GFP-PYR1* alone (Fig. 5c).

In addition, confocal microscopy showed that in root cells, ABA induced GFP-PYR1-Ub relocalization to intracellular puncta associated with the ER or with the plasma membrane (Fig. 5d,e). Quantitative analyses demonstrated that more puncta on – or close to – the plasma membrane can be found in GFP-PYR1-Ub cells than in GFP-PYR1 cells. Moreover, it was found that ABA treatment increased the microsome localization of both GFP-PYR1 and GFP-PYR1-Ub, GFP-PYR1-Ub had a higher accumulation ratio in the cell membrane after ABA treatment (Fig. 5f,g). These results suggested that PYR1 mono-ubiquitination and may be also mono-ubiquitination of other members of the ABA receptor family might enhance ABA perception by driving more receptor molecules through the intracellular membrane system. These receptor molecules ultimately converge on the puncta on/close to the plasma membrane, and lead to enhanced ABA sensitivity.

Gain-of-function of DOA10A mediates mono-ubiquitination of ABA receptor and enhances the sensitivity to ABA

Based on the results described so far, it is possible to speculate that the ABA-sensitive phenotype observed in *doa10a-1* mutants could be due to an increase in the mono-ubiquitination of ABA receptors such as PYR1. To further confirm the role of DOA10A-mediated mono-ubiquitination of ABA receptors within the ABA signaling pathway, we took advantage of an *in vivo* reconstructed ABA pathway (Fujii *et al.*, 2009). We thus cotransfected into *Arabidopsis* protoplasts the constructs coding for the core components of the ABA signal pathway as well as the *RD29B::LUC* reporter to reconstruct the ABA signal transduction. Indeed, in the presence of PYR1, ABI1 and SnRK2.6, the addition of ABA could activate the transcription of the *RD29B::LUC* reporter (Fig. 6a). When PYR1 was replaced by the PYR1-K14/63R double variant (which disrupts its mono-ubiquitination), ABA could still induce *LUC* expression, but to a lower level (Fig. 6a). When PYR1 was replaced by PYR1-Ub, ABA-induced *LUC* expression had a 2.5-fold increase. Furthermore, when DOA10A was cotransfected with PYR1, ABA-induced *LUC* expression was similar to that induced by ABA when PYR1-Ub alone was used (Fig. 6a). These results further confirm that ABA-induced signaling is enhanced by PYR1 mono-ubiquitination mediated by DOA10A.

Taken together, these results demonstrate that DOA10A mediates the mono-ubiquitination of the ABA receptor PYR1 – and likely of also other members of the ABA receptors family – driving it to the membrane puncta on/close plasma membrane through the endomembrane system, and that this leads to enhanced ABA sensitivity (Fig. 6b).

Discussion

The RING E3 ubiquitin ligase DOA10 has been first identified in *Saccharomyces cerevisiae*, during genetic screens aiming at uncovering regulators of the degradation of Matα2, a short-lived transcriptional repressor (Swanson *et al.*, 2001). DOA10 was found to localize to the ER and regulate the ER unfolded protein response in yeast (Swanson *et al.*, 2001). Interestingly, DOA10 is conserved from lower to higher eukaryotes. In human, the DOA10 ortholog TEB4/MARCH6 is required for the degradation of both Type 2 iodothyronine deiodinase and squalene monooxygenase, thus affecting both thyroid hormone signaling and cholesterol synthesis (Zavacki *et al.*, 2009; Zelcer *et al.*, 2014). In *Arabidopsis*, the DOA10 ortholog CER9, named DOA10A in this study, was initially identified through the semi-glossy inflorescence stem phenotype of its corresponding mutant (*cer9-1*; Koornneef *et al.*, 1989). The CER9 RING domain shares a high amino acid identity percentage with that of DOA10 (49%) and TEB4 (57%). CER9 was shown to be involved in wax and cutin synthesis process, as *cer9-1* leaves have a thicker cuticle membrane, leading to lower transpiration rates and drought tolerance (Lü *et al.*, 2012). The positive role of DOA10A in drought tolerance is also indicated by its identification in a genetic screen aiming at isolating suppressors of the *drought hypersensitive 2* (*dry2*) mutant; the *suppressor of dry2 defects 1* (*sud1*) mutant carries in fact a mutation in the *DOA10A* gene and can rescue most *dry2* phenotypical defects by affecting 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) activity (Doblas *et al.*, 2013). Previous study researched the structure of DOA10-like protein, conducted homologous phylogenetic tree

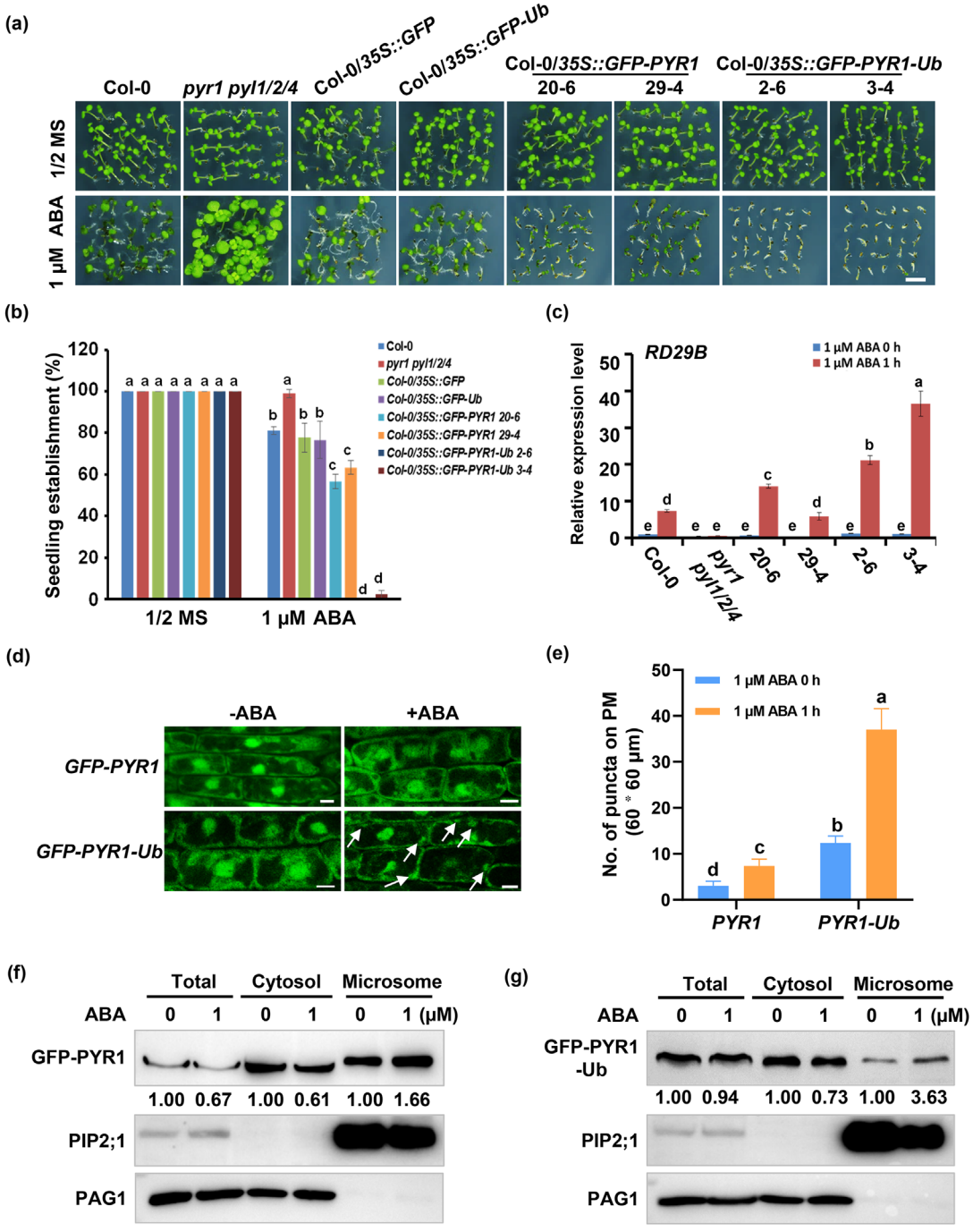

Fig. 5 Overexpression of a monoubiquitinated mimic of PYR1 enhances abscisic acid (ABA) sensitivity. (a, b) Overexpression of *GFP-PYR1-Ub* increases wild-type sensitivity to ABA-mediated inhibition of seedling establishment. Seeds were sown on 1/2 MS medium with or without 1 μM ABA. Seedlings were photographed (a) and the seedling establishment rates were determined (b) at 12 d post sowing. Plants overexpressing *GFP* and *GFP-Ub* were used as controls. Bar, 0.5 cm. Error bars represent the SD (triplicate measurements; $n = 30$). Different letters indicate significantly different groups ($P < 0.05$, ANOVA, Tukey's HSD). (c) Overexpression of *GFP-PYR1-Ub* enhances ABA-mediated *RD29B* expression. RNA was extracted from 10-d-old seedlings treated with or without 1 μM ABA for 1 h. *ACTIN2* was used as the internal control. Error bars represent the SD (triplicate measurements). (d) GFP-PYR1-Ub localizes to membrane compartments under ABA treatment. Seeds were sown on 1/2 MS, grown vertically for 5 d, and incubated in liquid medium with or without 1 μM ABA for 1 h. Arrows indicate the localization of the GFP-PYR1-Ub fusion protein. Bar, 5 μm. (e) Quantification of the vesicle puncta formed by GFP-PYR1-Ub in (d). Error bars represent the SD (triplicate measurements). Different letters indicate significantly different groups ($P < 0.05$, ANOVA, Tukey HSD). (f, g) ABA treatment promotes GFP-PYR1 and GFP-PYR1-Ub to localize to membrane compartments. Seedlings were grown on MS plate vertically for 10 d, and then were incubated in liquid medium with or without 1 μM ABA for 1 h. The total, cytosol and microsome fractions were isolated separately. Immunoblot assays with anti-GFP antibody were performed to detect GFP-PYR1 (f) and GFP-PYR1-Ub (g). PIP2;1 was used as the membrane marker and PAG1 (20S proteasome a-subunit G1) as a cytosol fraction marker. ImageJ was used for gray scale analysis, and the ratio between the amount of GFP fusion protein and marker protein at the initial time point was set to 1.

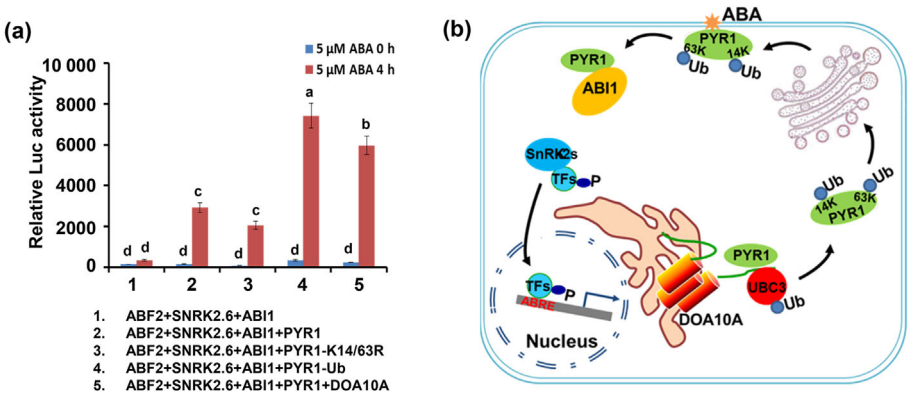

Fig. 6 DOA10A mediates mono-ubiquitination of abscisic acid (ABA) receptor and enhances seedling sensitivity to ABA. (a) The core ABA signal transduction was reconstructed in *Arabidopsis* protoplasts. *Promoter-RD29B::LUC* was used as the ABA-responsive reporter and *Promoter-ZmUBQ::GUS* was used as the internal control. After 24-h transfection, protoplasts were treated with or without 5 μM ABA for 4 h under light. Different combinations of constructs were transfected as indicated. Error bars represent the SD (triplicate measurements). Different letters indicate significantly different groups ($P < 0.05$, ANOVA, Tukey's HSD). (b) A working model for DOA10A function. DOA10, in conjunction with the E2 conjugase UBC3, mono-ubiquitinates ABA receptor PYR/PYL family members on selected amino acids (14^{th}K and 63^{rd}K for PYR1). Mono-ubiquitinated PYR1 (on 14^{th}K and 63^{rd}K) might relocate to the membrane puncta on/close plasma membrane through the endomembrane system, leading to enhance the sensitivity to ABA.

analysis and found that DOA10 mutants were sensitive to ABA, but did not deeply research how DOA10 responded to ABA signals. In addition, the result found that AtDOA10s participated in homeostasis regulation of sterol biosynthesis by controlling the conversion of AtSQE1 (Etherington *et al.*, 2023). Thus, not only the DOA10 amino acid sequence has been conserved throughout evolution but also DOA10 molecular function.

Since the *SUD1/DOA10A* mutation can rescue the ABA insensitivity of *dry2* mutant stomatal response, it was suggested that *SUD1*/DOA10A could be involved in the ABA signaling pathway (Doblas *et al.*, 2013). However, how and why ERAD is involved in plants stress responses is less understood. It has been proposed that a stressful environment might disrupt the folding process of some specific proteins, whose subsequent degradation by ERAD could trigger plants stress responses (Liu & Howell, 2010). In this work, we aimed at investigating the interaction between ERAD and ABA signaling pathways. We report here that the *doa10a-1* mutant is indeed hypersensitive to ABA and that DOA10A is a functional E3 ubiquitin ligase in plants. It should be noted that DOA10A exhibits strong E3 activity only when combined with the *Arabidopsis* E2 counterparts of yeast UBC2 (UBC1, UBC2 and UBC3 in *Arabidopsis*), while in yeast, DOA10 E3 activity requires yeast UBC6 and UBC7 (Ruggiano *et al.*, 2014). This different E2 specificity in plants from yeast may be the reason why until now there was no direct evidence for a DOA10A E3 activity in plants. In addition, because UBC6 and UBC7 but not UBC2 are known to be important ERAD components in yeast, the discovery of a functional DOA10A/UBC1/2/3 module in *Arabidopsis* might imply that DOA10A has additional unique function in plants, besides ERAD. Also, whether UBC1/2/3 in *Arabidopsis* act in ERAD system to counteract the ER stress is an interesting question needs to be resolved.

In the course of our work, another group reported that the ABA hypersensitive phenotype of *cer9-2* (corresponding to our *doa10a-1* mutant) could be rescued by the ABA biosynthesis mutant *aba2* and by the ABA biosynthesis inhibitor fluridone; as ABA concentration was increased in *cer9-2* mutant seeds compared to their WT controls, the authors concluded that DOA10A/CER9 affected ABA response through the regulation of ABA biosynthesis (Zhao *et al.*, 2014). However, we observed that *doa10a-1* seedlings germinated and grew normally on the medium without exogenous ABA, suggesting that their high endogenous ABA concentration may not be the primary reason of their hypersensitivity to exogenous ABA. Indeed, we show here that *doa10a-1* mutation can rescue the ABA-insensitive phenotype of an ABA receptor quadruple mutant. It has been reported that ABA receptor family includes 14 members, which causes functional redundancy (Ma *et al.*, 2009; Park *et al.*, 2009). As a truncated form of DOA10A, $DOA10A^{1-150}$ is able to mediate ubiquitination of all 14 ABA receptor members, it is very likely that all PYR/PYL type ABA receptors can be ubiquitinated by the DOA10A-1 mutant protein expressed in the *doa10a-1* mutant. We can thus conclude that the DOA10A is involved in the ABA signaling pathway mainly because it mediates ubiquitination of the ABA receptors.

What is the fate of the ABA receptors after ubiquitination? Several groups reported a few E3 ligases – such as RSL1, $CRLL4^{DDA1}$ and the APC E3 complex activator TE – are able to facilitate the degradation of ABA receptors through poly-ubiquitination (Bueso *et al.*, 2014; Irigoyen *et al.*, 2014; Lin *et al.*, 2015). Different from the above studies, we found that DOA10A is able to mediate mono-ubiquitination of specific amino acids of the ABA receptors. According to previous researches in yeast and animals, mono-ubiquitination may affect protein localization, activity and interaction (Winston *et al.*, 1999; Li *et al.*, 2003; Bienko *et al.*, 2005), rather than degradation. We could indeed show that a transiently expressed PYR1 mono-ubiquitination mimic (Terrell *et al.*, 1998) localized to the endomembrane system and moved to plasma membrane upon ABA treatment, causing ABA hypersensitivity. Thus, DOA10A plays a sophisticated role in the ABA signaling

pathway, by mediating mono-ubiquitination of ABA receptors, driving them to the membrane and enhancing ABA perception. It should be noted that it cannot be excluded that the mono-ubiquitination of ABA receptor affects their binding to ABA or PP2C. Additionally, mono-ubiquitination could facilitate the degradation of the ABA receptor after it has fulfilled its role in perceiving and transmitting the ABA signal. These aspects warrant further investigation. In conclusion, this study together with previous studies on the ubiquitination of PYR/PYL-type ABA receptors, indicate that both mono- and polyubiquitination modulate the function of ABA receptors by regulating their stability and their subcellular localization. Understanding how the activities of E3 ubiquitin ligases such as DOA10A, RSLs, $CRLL4^{DDA1}$ and the APC E3 complex activator TE function together to control the different ubiquitinated forms of ABA receptors, and thus, the amplitude and specificity of ABA signaling still awaits further exploration.

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Competing interests

None declared.

Author contributions

FY and QX conceived the project. FY, QX and QL designed the research procedure. CL, QL, ZS, RX, QC, XL and YD performed the experiments. CL, QL, FY, SY and QX analyzed the data. QL, FY and QX wrote the manuscript with input from all authors. FY, QX and GS revised the manuscript. CL and QL contributed equally to this work.

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Data availability

All data that support the results of this article are contained within the article and its Supporting Information (Figs S1–S7; Tables S1–S3).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Abscisic acid response of *Arabidopsis* mutants of putative orthologs of yeast endoplasmic reticulum-associated degradation components.

Fig. S2 *doa10a-1* is a gain-of-function mutant.

Fig. S3 Overexpression of the first N terminal 150 amino acids of DOA10A enhances abscisic acid sensitivity.

Fig. S4 The *doa10a-1* mutation enhances the abscisic acid-mediated inhibition of seedling establishment of the *pyr1 pyl1/2/4 quadruple* mutant.

Fig. S5 DOA10A interacts with and ubiquitinates all PYR/PYL family members.

Fig. S6 Colocalization of PYR1 with subcellular compartment markers.

Fig. S7 Protein levels of PYR1 and PYR1-Ub in transgenic lines.

Table S1 Summary of used primers.

Table S2 *Arabidopsis* putative orthologs of endoplasmic reticulum-associated degradation genes from *Saccharomyces cerevisiae*.

Table S3 Summary of genes used in the ubiquitination assays.

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