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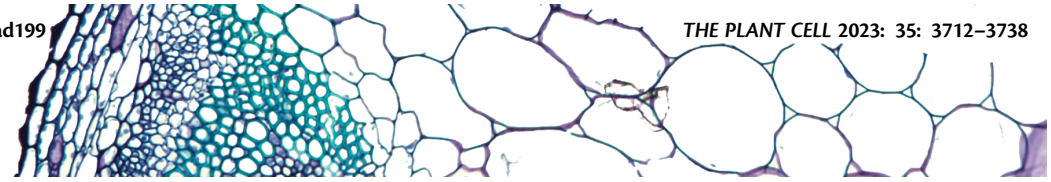

Research Article

The Arabidopsis F-box protein SKP1-INTERACTING PARTNER 31 modulates seed maturation and seed vigor by targeting JASMONATE ZIM DOMAIN proteins independently of jasmonic acid-isoleucine

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Abstract

F-box proteins have diverse functions in eukaryotic organisms, including plants, mainly targeting proteins for 26S proteasomal degradation. Here, we demonstrate the role of the F-box protein SKP1-INTERACTING PARTNER 31 (SKIP31) from Arabidopsis (*Arabidopsis thaliana*) in regulating late seed maturation events, seed vigor, and viability through biochemical and genetic studies using *skip31* mutants and different transgenic lines. We show that *SKIP31* is predominantly expressed in seeds and that SKIP31 interacts with JASMONATE ZIM DOMAIN (JAZ) proteins, key repressors in jasmonate (JA) signaling, directing their ubiquitination for proteasomal degradation independently of coronatine/jasmonic acid-isoleucine (JA-Ile), in contrast to CORONATINE INSENSITIVE 1, which sends JAZs for degradation in a coronatine/JA-Ile dependent manner. Moreover, JAZ proteins interact with the transcription factor ABSCISIC ACID-INSENSITIVE 5 (ABI5) and repress its transcriptional activity, which in turn directly or indirectly represses the expression of downstream genes involved in the accumulation of LATE EMBRYOGENESIS ABUNDANT proteins, protective metabolites, storage compounds, and abscisic acid biosynthesis. However, SKIP31 targets JAZ proteins, deregulates ABI5 activity, and positively regulates seed maturation and consequently seed vigor. Furthermore, ABI5 positively influences *SKIP31* expression, while JAZ proteins repress ABI5-mediated transactivation of *SKIP31* and exert feedback regulation. Taken together, our findings reveal the role of the SKIP31-JAZ-ABI5 module in seed maturation and consequently, establishment of seed vigor.

Introduction

Seed maturation includes the arrest of embryo morphogenesis, the acquisition of desiccation tolerance, and the accumulation of storage reserves and protective metabolites. It involves a complex regulatory network that ultimately improves seed survival and vigor upon maturity (Probert et al. 2007; Verdier et al. 2013; Righetti et al. 2015). The phytohormone abscisic acid (ABA) and the ABA-responsive master regulators ABA-INSENSITIVE 3 (ABI3), ABA-INSENSITIVE 5 (ABI5), FUSCA3 (FUS3), and LEAFY COTYLEDON 1 (LEC1) play a key role in regulating seed maturation (Bäumlein et al. 1994; Parcy et al. 1997; Raz et al. 2001; To et al. 2006; Kagaya et al. 2005; Santos-Mendoza et al. 2008; Sugliani et al. 2009). Particularly, Arabidopsis (*Arabidopsis thaliana*) loss-of-function single mutants of *ABI3* or *LEC1* produce seeds that lose their viability during maturation drying or the first few weeks after harvest (Ooms et al. 1993; Nambara et al. 1994; Sugliani et al. 2009; Delahaie et al. 2013). The stability and activities of these ABA master regulators are regulated

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Background: Seed maturation is a highly complex physiological program during which seeds acquire adaptive features such as seed desiccation tolerance and deposition of seed storage reserves. These features allow seeds to extend their viability and germinability while remaining in a desiccated state for long periods of time. To achieve this feat, seeds engage multilayered regulatory networks that activate many genes involved in various mechanisms that ultimately improve seed survival and vigor upon maturity. Among these networks, the phytohormone abscisic acid (ABA) and ABA-responsive master regulators play a key role in modulating seed maturation.

Question: The regulatory role of JASMONATE ZIM DOMAIN (JAZ) proteins was recently reported in repressing ABA signaling by suppressing ABSCISIC ACID INSENSITIVE3 and ABSCISIC ACID-INSENSITIVE 5 (ABI5) transcriptional activity during germination. However, how ABI-related transcription factors are repressed by JAZ and how the ABA signaling is derepressed during seed maturation are unclear.

Findings: We identified the SKIP31–JAZ–ABI5 module as a regulator of seed maturation and seed vigor in Arabidopsis. We show that SKIP31, an F-box protein, targets JAZ proteins for proteasomal degradation in a jasmonate (JA)-isoleucine (Ile)-independent manner to alleviate the inhibition imposed by JAZ proteins on ABI5. ABA-mediated downstream signaling thus becomes activated, which is essential for seed maturation, desiccation tolerance, and establishment of seed vigor and viability.

Next step: We will test whether other transcription factors and/or regulatory proteins besides ABI5 contribute to the SKIP31–JAZ–ABI5 module to regulate seed maturation. Additionally, we will ask if SKIP31 influences JA-Ile-dependent regulation of the JAZ–MYELOCYTOMATOSIS (MYC) module in the JA signaling pathway and JA responses.

by interaction and crosstalk with several other transcriptional activators and repressor proteins (Meurs et al. 1992; Liu and Stone 2010; Leprince et al. 2017).

Recent studies have revealed the repression of ABI3 and ABI5 transcriptional activity by JASMONATE ZIM DOMAIN (JAZ) proteins in wheat (*Triticum aestivum*) and Arabidopsis (Ju et al. 2019; Pan et al. 2020; Varshney and Majee 2021). Furthermore, the JAZ–ABI3/ABI5 module integrates the jasmonate (JA) and ABA signaling pathways and is involved in the regulation of seed germination (Ju et al. 2019; Pan et al. 2020; Varshney and Majee 2021). However, how this JAZ-mediated repression of ABA-related transcription factor (TF) activity is released during seed maturation is not known. In general, JAZ proteins function as repressors in JA signaling by inhibiting the activity of several TFs, including MYELOCYTOMATOSIS (MYCs) and MYELOBLASTOSIS (MYBs), causing the downregulation of JA-responsive gene expression. CORONATINE INSENSITIVE 1 (COI1), an F-box protein, promotes the degradation of JAZ proteins in a JA-Ile-dependent manner by employing the ubiquitin proteasome pathway (Xie et al. 1998; Xu et al. 2002; Chini et al. 2007; Thines et al. 2007; Yan et al. 2013). Twelve JAZ members have been identified in Arabidopsis and have been revealed to function in pathways affecting seed germination, trichome development, anther and pollen formation, and defense against herbivores and insect pests (Niu et al. 2011; Schweizer et al. 2013; Cheng et al. 2014; Qi et al. 2015; Pan et al. 2020). Moreover, a recent study unveiled the role of JAZ proteins in controlling seed size in a JA-dependent manner (Hu et al. 2021).

The mechanisms that control seed maturation, mediated by ABA signaling in particular, are complex mainly due to the crosstalk between phytohormones as well as the involvement of the 26S proteasome pathway, which essentially controls almost all phytohormone signaling pathways. Indeed, the ubiquitin proteasome pathway has emerged as a central player in the regulation of nearly every aspect of plant biology (Hershko and Ciechanover 1998; Moon et al. 2004; Smalle and Vierstra 2004; Vierstra 2009; Santner and Estelle 2010; Varshney and Majee 2022). It employs 3 key enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3) to modify target proteins via ubiquitination. Most ubiquitinated target proteins are degraded by the 26S proteasome complex (Hatfield et al. 1997; Pickart 2001; Hershko and Ciechanover 1998; Vierstra 2009). Among E3 ligase enzymes, the SKP1-Cdc53-F-box protein (SCF) complex, consisting of SKP1, CULLIN1/CDC53, the ring finger protein RING-BOX 1 (also named HRT and REGULATOR OF CULLINS-1), and an F-box protein, is highly diverse and well-studied. F-box proteins recognize and tag substrates for modification and/or elimination, thus determining substrate specificity. About 700 F-box proteins have been predicted to be encoded by the Arabidopsis genome; of them, only a few F-box proteins have been characterized along with their substrates (Gagne et al. 2002; Jin et al. 2004).

We previously discovered that the F-box protein SKP1-INTERACTING PARTNER 31 (SKIP31) interacts with ARABIDOPSIS SKP1-LIKE PROTEIN 13 (ASK13), which has a positive effect on seed germination, vigor, and seedling

growth, particularly under abiotic stress conditions (Rao et al. 2018). Although SKIP31 can ubiquitinate 14-3-3 (χ isoform) in vitro (Hong et al. 2017), its functional role has not yet been explored in detail.

Here, we show that SKIP31 is a bona fide F-box protein whose encoding gene is highly expressed in seeds, particularly during the late maturation phase. We demonstrate that a *skip31* mutant and RNA interference (RNAi) lines show compromised seed maturation, with aberrant seed morphology, lower seed germination, and viability as dry seeds; seed-specific overexpression (OE) of *SKIP31* resulted in greater seed germination vigor and viability. We establish that SKIP31 interacts with JAZ proteins in a coronatine (COR)/JA-Ile-independent manner, unlike the other F-box protein COI1, by yeast 2-hybrid (Y2H), bimolecular fluorescence complementation (BiFC), and in vitro pull-down assays. We further show that SKIP31 ubiquitinates JAZs and mediates their degradation via the 26S proteasome. We discovered that JAZs negatively regulate the transcriptional activity of ABI5, downregulating the expression of several genes required for seed maturation, desiccation tolerance, ABA responses, and seed storage accumulation, directly or indirectly. Notably, SKIP31 activates ABI5 by targeting JAZ proteins for degradation, explaining its contribution to seed maturation, seed desiccation tolerance, seed germination, and vigor.

Results

SKIP31 is highly expressed in seeds, and its encoded protein interacts with ASK proteins

SKIP31 (At5g45360) encodes a 316-amino acid (aa) F-box protein containing a characteristic F-box domain at its N terminus (aa 63 to 119) and a herpes UL92 domain at the C terminus (aa 252 to 256) (Supplemental Fig. S1). We previously showed that SKIP31 interacts with ASK13 and confirmed it as a bona fide F-box protein (Rao et al. 2018). To test whether SKIP31 also interacts with other ASKs, we performed a Y2H assay with other ASK proteins (ASK1 to ASK15, ASK17, ASK18, ASK19) in addition to ASK13. We determined that SKIP31 can indeed interact with most ASK members (ASK1, ASK2, ASK4, ASK5, ASK6, ASK8, ASK11, ASK13, ASK17, and ASK18) (Fig. 1A).

To investigate the possible function of SKIP31, we analyzed *SKIP31* transcript levels in different organs in Arabidopsis by reverse transcription quantitative PCR (RT-qPCR). Similar to data from the TAIR-eFP browser (https://www.arabidopsis.org/), we detected *SKIP31* transcripts predominantly in seeds, with lower expression in roots, stems, leaves, and flowers (Fig. 1B). To more precisely delineate *SKIP31* expression in seeds, we examined the spatiotemporal activity of the *SKIP31* promoter by generating *proSKIP31:GUS* (*ß-glucuronidase*) stable transgenic lines in Arabidopsis. GUS staining revealed *SKIP31* expression in all vegetative and reproductive tissues tested including seeds (Supplemental Fig. S2A; Fig. 1, C to I). A detailed analysis of microtome seed sections indicated that *SKIP31* is expressed in all parts of mature seeds, including the embryonic layer, the endosperm layer, and the seed coat (Fig. 1C II to V).

To gain a detailed representation of *SKIP31* transcript accumulation during seed development, we marked Arabidopsis flowers from 1 d after flowering (DAF; S1) to the formation of mature seeds (S7), in 3-d intervals. We detected low relative *SKIP31* transcript levels during early the seed developmental stages S1 to S4 (0 to 12 DAF), followed by a sharp increase in transcript abundance from S5 (13 to 15 DAF) to S7 (19 to 21 DAF), corresponding to the seed maturation phase (Fig. 1D; Supplemental Fig. S2B). *SKIP31* transcript levels gradually decreased as seed germination proceeded (Fig. 1E; Supplemental Fig. S2C). To check whether *SKIP31* expression is also influenced by phytohormones and abiotic stress, we measured *SKIP31* transcript levels in seedlings treated with the indicated phytohormones or abiotic stresses. *SKIP31* transcript accumulation rose to various extents in the presence of different phytohormones and abiotic stress; however, we observed a maximum induction under ABA and dehydration treatments (Fig. 1, F and G).

Subcellular localization studies of SKIP31 through transient expression of *SKIP31-YFP* (encoding SKIP31 fused to yellow fluorescent protein) in *Nicotiana benthamiana* leaves revealed its localization in the nucleus (Fig. 1H). Together, our results demonstrate that SKIP31, a nucleus-localized F-box protein, interacts with ASK proteins and that the *SKIP31* transcript accumulates predominantly in the late stages of seed maturation and is significantly induced in response to ABA and dehydration treatment.

skip31 T-DNA insertion mutants and RNAi lines show a defect in seed maturation and germination vigor and/or viability, while seed-specific OE lines show improved seed vigor in Arabidopsis

To investigate the biological role of SKIP31, we analyzed *skip31* T-DNA insertion lines (CS848440 and CS829396). The 2 mutants harbored a T-DNA insertion in the 3′ untranslated region (3′ UTR) and accumulated significantly less *SKIP31* transcript than wild-type (WT) (Supplemental Fig. S3A). We did not observe any obvious differences in terms of overall growth or development of these mutants compared to WT under normal growth conditions. However, mutant seeds (4-wk-after harvesting) exhibited a significantly lower seed germination rate (~60% to 70%) compared to WT (~98% to 100%) on water agar as well as on half-strength MS medium (Fig. 2A; Supplemental Fig. S3, B to D). To check whether the lower germination of these mutant seeds was related to their viability, we performed tetrazolium staining and quantified formazan accumulation. Mutant seeds showed less red coloration following tetrazolium staining (Supplemental Fig. S3E), which was consistent with the lower absorbance measured for formazan accumulation relative to WT seeds, indicative of the poor viability of the mutant seeds (Fig. 2B).

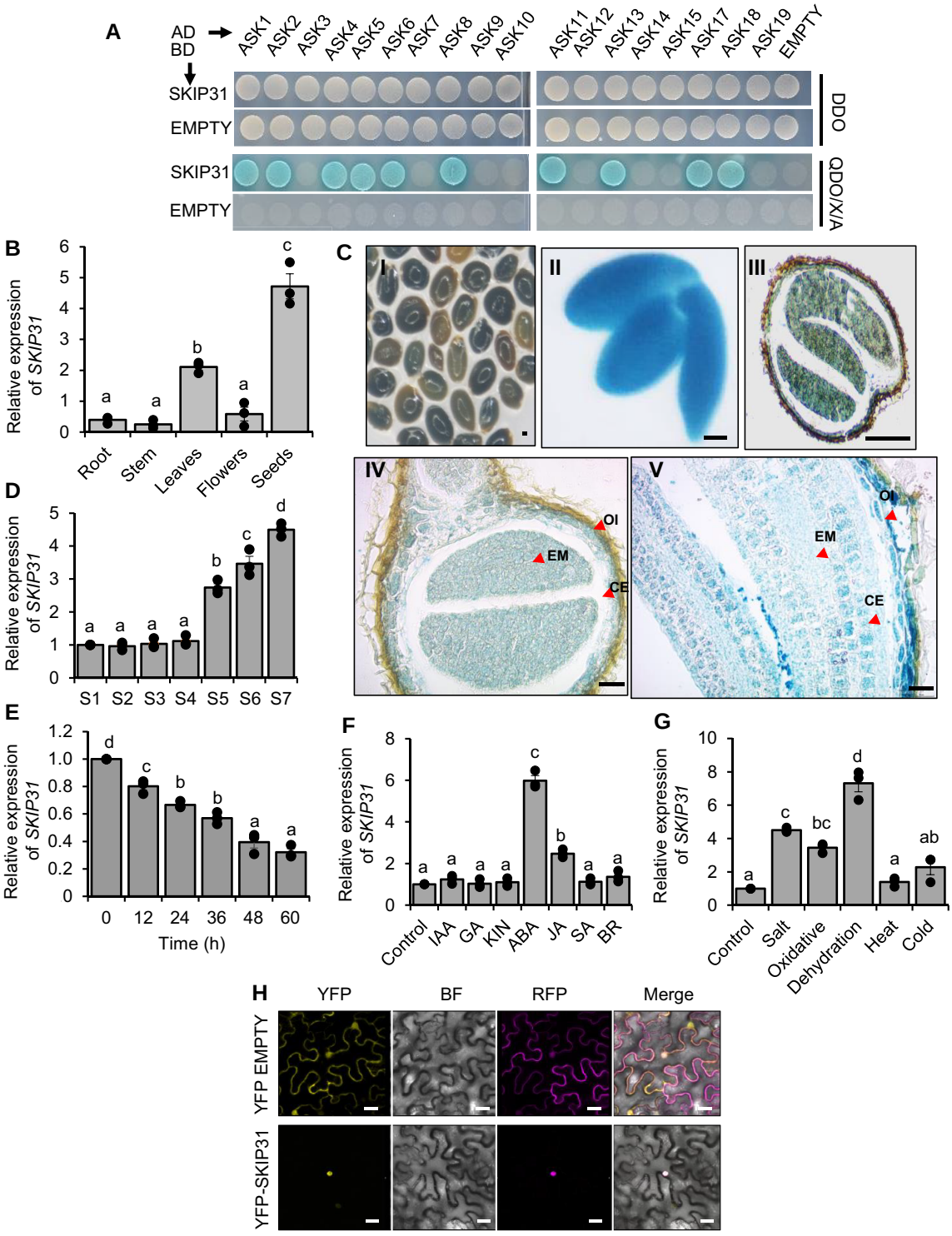

Figure 1. SKIP31 interacts with ASK proteins, and *SKIP31* is predominantly expressed in seeds. **A)** Y2H assay showing the interaction between SKIP31 and the indicated ASK proteins. Yeast cells cotransformed with BD-SKIP31, and the indicated AD-ASKs were grown on yeast synthetic double-drop-out (DDO) medium lacking leucine and tryptophan (upper panel) and quadruple-drop-out (QDO) medium lacking leucine, tryptophan, histidine, and adenine containing X-α-gal/aureobasidin agar (QDO/X/A) for 3 d (lower panel). The empty vectors BD and AD were used as a negative control. **B)** RT-qPCR analysis of *SKIP31* transcript levels in different Arabidopsis tissues. **C)** Histochemical *ß-glucuronidase* (GUS) staining in seeds of Arabidopsis *proSKIP31:GUS* transgenic lines. I, mature dry seeds; II, seed embryo; III, transverse section of seed; IV, longitudinal section of seed; V, enlarged view of a transverse section of seed. Scale bars, 100 μm (I to III), 20 μm (IV to V). **D to G)** RT-qPCR analysis of *SKIP31* transcript levels **D)** during seed development. Seed developmental stages (SI to S7) based on DAF are as follows: S1, 0 to 3 DAF; S2, 4 to 6 DAF; S3, 7 to 9 DAF; S4, 10 to 12 DAF; S5, 13 to 15 DAF; S6, 16 to 18 DAF; S7, 19 to 21 DAF. **E)** During seed germination. The seeds were hydrated in water on

(continued)

To confirm the poor germination and viability observed in *skip31* mutant seeds, we generated intron-spliced hairpin-RNAi lines and seed-specific OE lines for *SKIP31* in Arabidopsis Col-0 (WT). We selected and confirmed the respective *SKIP31*-OE and *SKIP31*-RNAi lines by checking the accumulation of visual markers (GUS in *SKIP31*-OE lines and DsRED in *SKIP31*-RNAi lines) and transcript levels via RT-qPCR (Supplemental Fig. S4, A to F). As with the *skip31* T-DNA mutants, 4-wk-old seeds of *SKIP31*-RNAi lines (RF1, RF2, RF3) also exhibited significantly lower germination rates (∼50%) on water agar and half-strength MS medium (Fig. 2C; Supplemental Fig. S5, A to C) and thus poor viability (Fig. 2D; Supplemental Fig. S5D). By contrast, *SKIP31*-OE lines (-OE1, -OE2, and -OE3) reached ∼98% to 100% germination and full viability (Fig. 2, A and B; Supplemental Fig. S5, A to D).

To explore whether the lower seed germination and viability of *skip31* mutant and *SKIP31*-RNAi lines were due to defects in seed development and/or seed maturation, or during storage, we carried out various phenotypic analyses from the S4 (10 to 12 DAF) to the S7 (19 to 21 DAF) stages, corresponding to the window of high *SKIP31* expression. Although we observed slightly reduced germination upon desiccation treatment (developing seeds [S5:13 to 15 DAF] were kept at 25 °C for 2 d at 15% relative humidity (RH) in seed storage cabinet) in *skip31* mutant (CS848440) and *SKIP31*-RNAi seeds compared to WT seeds at the S5 stage (Supplemental Fig. S6), we observed no significant alteration in terms of seed development or germination patternuntil S5 (12 to 15 DAF), when the embryo is fully formed and acquires germinability (Supplemental Fig. S7, A and B). Interestingly, we observed premature browning, shrunken seeds, and lower germination potential during the late seed maturation stage (16 to 21 DAF) for the *skip31* mutants and *SKIP31*-RNAi lines compared to WT and *SKIP31*-OE (Supplemental Fig. S7, C and D). We investigated seed morphological phenotypes in all indicated genotypes via scanning electron microscopy (SEM). As shown in Fig. 2E, dry mature seeds from the *skip31* mutant (CS848440) and *SKIP31*-RNAi liens showed pronounced abnormalities, as evidenced by shrunken and distorted shapes, possessing irregularly shaped epidermal cells, nonuniform mucilage pockets, and columella. By contrast, WT and *SKIP31*-OE seeds showed a typical seed morphology, exhibiting hexagonal epidermal cells with a volcano-shaped structure (the columella) in the center of each cell.

We measured the germination rate of freshly harvested (0-wk-old [0W]) to 8-wk-old (8W) postharvested seeds (0W, 2W, 4W, and 8W) of all the indicated genotypes. We determined that at 0W, the mutant CS848440 and *SKIP31*-RNAi seeds experience a ∼10% to 20% drop in germination ability relative to WT, and the germination rate of this mutant and *SKIP31*-RNAi lines progressively decreased with longer storage duration much more rapidly than WT. For instance, at 2W from the *SKIP31*-RNAi line reached about ∼70% to 75% germination, while 4W and 8W *SKIP31*-RNAi seeds showed a further decrease in germination, as low as ∼50%, compared to ∼95% to 100% germination in WT (Fig. 2F).

To further check that the disruption of *SKIP31* is responsible for the observed seed phenotypes, we overexpressed *SKIP31* into CS848440 mutant plants and subsequently examined *SKIP31* expression via RT-qPCR (Supplemental Fig. S8A). We observed that the mutant plants overexpressing *SKIP31* show a full rescue of the defects seen in the CS848440 mutant for seed germination, viability, and seed morphology (Fig. 2, G and H; Supplemental Fig. S8, B to D).

To support the involvement of SKIP31 in regulating seed germination vigor, we conducted controlled deterioration treatments (CDTs) as it has been commonly used to evaluate seed vigor. Accordingly, we subjected *SKIP31*-OE and *SKIP31*-RNAi seeds along with WT to CDT (45 °C and 70% RH) for 4 d and recorded the resulting germination rate and seed viability. Under normal conditions (0-d CDT), 4-wk-old *SKIP31*-OE and WT seeds showed 100% germination, whereas *SKIP31*-RNAi seeds showed significantly lower germination (∼50% germination). Seeds from all genotypes progressively deteriorated as the duration of the CDT increased. After 4 d of CDT, WT, and *SKIP31*-RNAi seeds achieved only about ∼0% to 2% germination, whereas *SKIP31*-OE seeds exhibited significantly greater germination rates (∼15% to 20%) and viability, which was strongly correlated with formazan absorbance and red coloration when subjected to tetrazolium staining before and after the CDT (Fig. 2, I and J; Supplemental Fig. S9, A and B).

To check whether the reduced germination phenotype of mature dry seeds of the *skip31* mutant and *SKIP31*-RNAi lines can be rescued by exogenous gibberellic acid (GA), we treated WT, *skip31* mutant, and *SKIP31*-RNAi seeds with 10 μM

(**Figure 1.** Continued)
Whatman filter paper in 35-mm Petri dishes from 0 to 60 h at 12-h intervals. **F)** For phytohormone treatment, 7-d-old Arabidopsis seedlings were treated with 10 μM of different phytohormones on half-strength MS agar medium: indole-3-acetic acid (IAA), GA, kinetin (KIN), ABA, methyl JA, salicylic acid (SA), and brassinosteroids (BR) for 12 h. **G)** Seven-day-old Arabidopsis seedlings were challenged to different abiotic stress treatments for 12 h on half-strength MS agar medium. Salt stress, 200 mM NaCl; dehydration stress, −0.5 MPa polyethylene glycol (PEG); oxidative stress, 2 μM paraquat; heat stress, 37 °C; cold stress, 4 °C. *SKIP31* expression was normalized to *ACTIN* 2 **B, F, and G)** or At4g12590 **D, E)** and calculated using the ΔCT **B)** or ΔΔCT method **D to G)**. Values are means ± standard error of 3 biological replicates. Significant differences among means (α= 0.01, as determined by ANOVA) are denoted by different lowercase letters. **H)** Subcellular localization of SKIP31 in *Nicotiana benthamiana* leaf epidermal cells using Agrobacterium-mediated transient expression of *YFP-SKIP31*. The *35S:YFP* plasmid was used as control. NLS-RFP was used as a nuclear marker; AtCESA1-RFP was used as a plasma membrane marker. Scale bars, 20 μm. OI, outer layer; CE, cellular endosperm; EM, embryonic region.

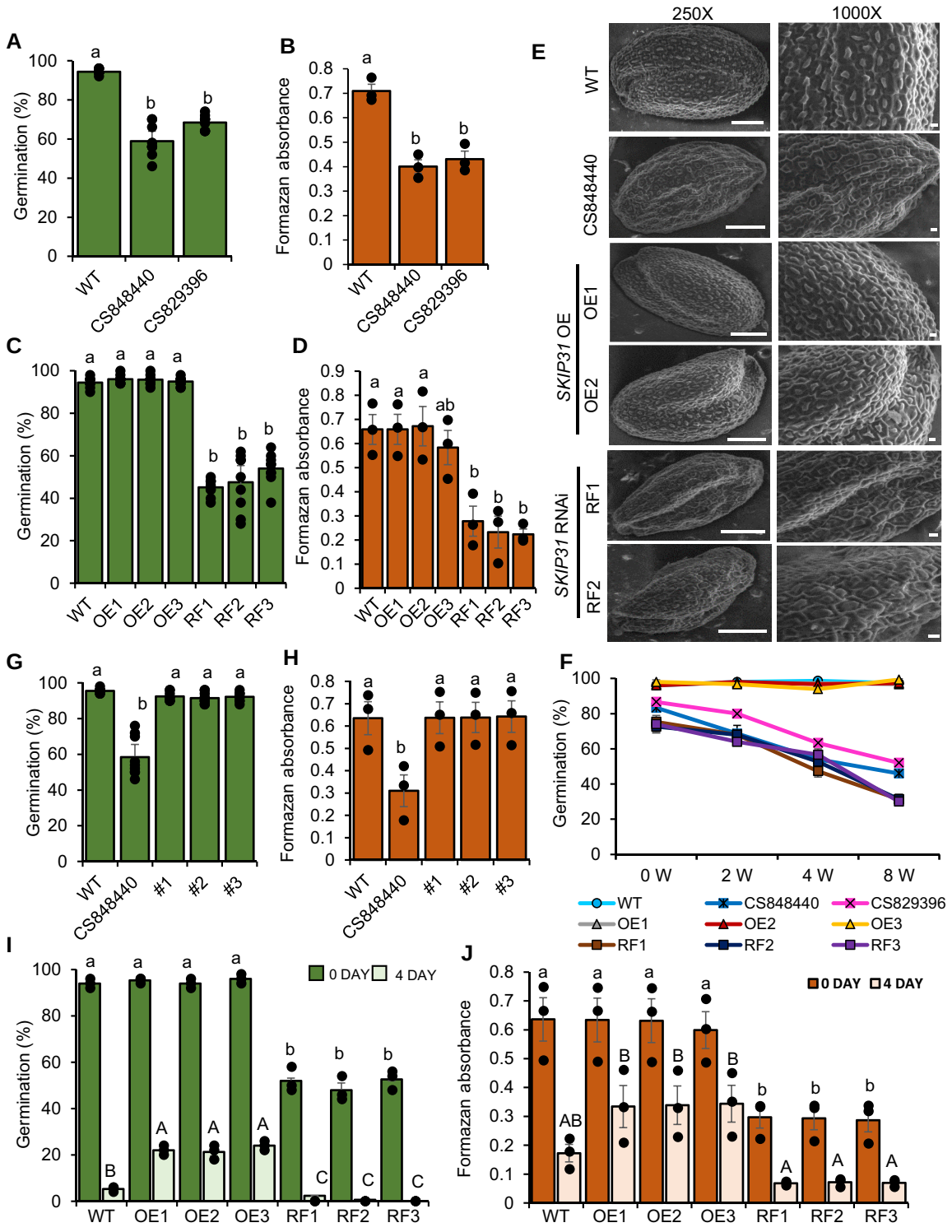

Figure 2. *skip31* T-DNA insertion mutants and RNAi lines show a defect in seed maturation, germination vigor, and viability, while seed-specific OE lines show improved seed vigor in Arabidopsis. For all seed germination experiments, mean and significance were calculated 3 biological replicates grown and harvested at 3 different times. For each biological replicate, all genotypes were grown in identical conditions and harvested at the same time, and seeds were pooled from at least 20 independent plants and each seed batch was tested at least 3 times as technical replicates. The value of each biological replicate is the mean of 3 technical replicates (50 seeds per technical replicate) or as indicated. Data from 3 biological replicates were analyzed by ANOVA. Different lowercase letters indicate significant differences ($\alpha = 0.01$ or as indicated in the legend). **A)** Seed germination rates (4-wk-old seeds) in WT, and the *skip31* mutants CS848440 and CS829396 on water-agar medium. **B)** Formazan absorbance upon tetrazolium staining of seeds from WT, and the *skip31* mutants CS848440 and CS829396. Seeds were incubated for 24 h in tetrazolium red at 28 °C and formazan

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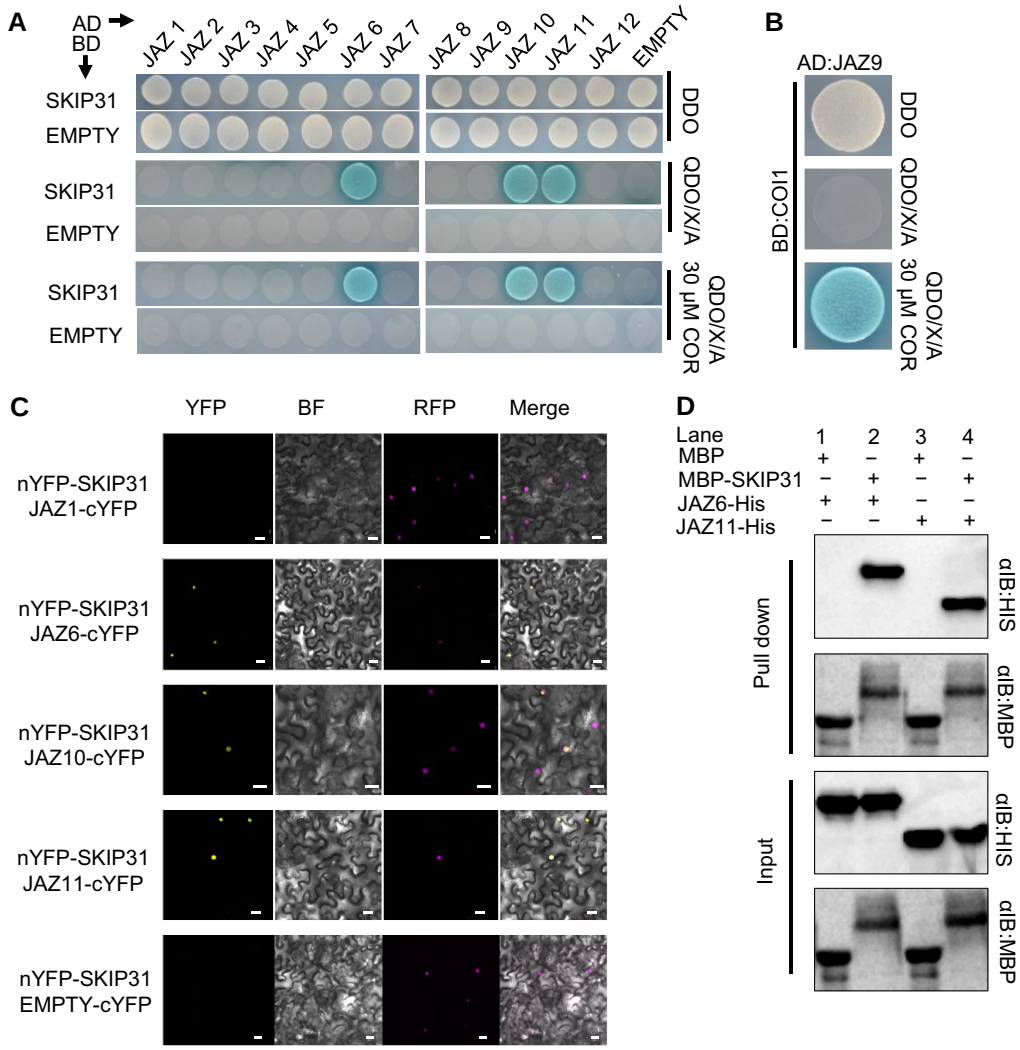

Figure 3. SKIP31 interacts with some JAZ proteins independently of COR. **A and B)** Y2H assay showing the interaction between SKIP31 and the indicated JAZ proteins. Yeast Y2H gold cells co-transformed with **A)** BD-SKIP31 and the indicated AD-JAZs (*JAZ1 to 12*); **B)** BD-COI1 and AD-JAZ9. All transformed cells were grown on DDO medium lacking leucine and tryptophan (top); QDO medium lacking leucine, tryptophan, histidine, and adenine and containing X-α-gal/aureobasidin agar (QDO/X/A) (middle); and QDO/X/A with 30 μM coronatine (COR) (bottom) for 3 d. Cells co-transformed with the empty vectors BD and AD were used as a negative control. **C)** BiFC assay in leaves of 1-month-old *N. benthamiana* plants using Agrobacterium-mediated transient expression. *nYFP-SKIP31* was coexpressed with the indicated *JAZ-cYFP*. *nYFP-SKIP31* was coinfiltrated with *cYFP* as control. *NLS-RFP* was co-infiltrated as a nuclear marker. Scale bars, 20 μm. **D)** In vitro MBP pull-down assay of recombinant purified JAZ6-His and JAZ11-His by MBP-SKIP31. MBP-SKIP31 was incubated with JAZ6-His or JAZ11-His; the resulting pulled down samples were examined by immunoblot analysis using anti-MBP (αIB-MBP) and anti-His (αIB-His) antibodies.

(**Figure 2.** Continued)
production was assayed by measuring the absorbance of 100 crushed seeds in 95% (v/v) ethanol at 492 nm. Formazan formation reflects seed vigor and/or viability. **C)** Seed germination rates (4-wk-old seeds) in WT, 3 independent *SKIP31* OE (OE1, -OE2, and -OE3), and *SKIP31*-RNAi (RF1, RF2, and RF3) lines. **D)** Formazan absorbance upon tetrazolium staining in WT, *SKIP31*-OE, and *SKIP31*-RNAi 4-wk-old seeds. **E)** Representative SEM micrographs of mature dry seeds for the indicated genotype, at magnifications 250× and 1,000×. Scale bars, 100 μm (250×), 10 μm (1,000×). **F)** Seed germination rates in WT, *skip31* mutants (CS848440 and CS829396), *SKIP31*-OE, and *SKIP31*-RNAi lines, from fresh (0W) to 8W postharvested seeds 7 d after stratification on water-agar medium. Values are means ± SE of 3 biological replicates. **G)** Seed germination rates (4-wk-old seeds) in WT, the CS848440 mutant, and CS848440 *SKIP31*-OE complementation lines (#1, #2, and #3). **H)** Formazan absorbance upon tetrazolium staining of seeds from WT, the CS848440 mutant, and CS848440 *SKIP31*-OE complementation lines (4-wk-old). Different lowercase letters indicate significant differences ($\alpha = 0.05$). **I)** Seed germination rates in seeds for WT, *SKIP31*-OE, and *SKIP31*-RNAi lines before (0 d) or after (4 d) CDT, 7 d after stratification on water-agar medium. **J**) Formazan absorbance upon tetrazolium staining of seeds from WT, *SKIP31*-OE, and *SKIP31*-RNAi lines before (upper panel, 0 d) and after (lower panel, 4 d) CDT. Different lowercase letters indicate significant differences ($\alpha = 0.01$, as determined by ANOVA). xW, x-wk-old postharvested seeds.

GA and assessed seed germination. Importantly, GA treatment did not affect germination in these genotypes (Supplemental Fig. S10). We conclude that the F-box protein SKIP31 participates in the late stages of seed maturation; lower *SKIP31* transcript levels affect seed morphology, germination potential upon maturity, and ultimately seed vigor and viability.

SKIP31 interacts with JAZ proteins in a JA-Ile/COR independent manner, and the Jas domain of JAZ proteins is required for the interaction with the C terminus of SKIP31

To investigate how SKIP31 participates in seed maturation and seed vigor, we wished to identify the potential substrate of SKIP31 by screening a normalized Arabidopsis Y2H cDNA library. After repeated screening and stringent selection, we identified JAZ6 and JAZ11 from the JAZ family as consistent interactive partners. To confirm the interaction between SKIP31 and these identified JAZ proteins and other JAZ family members (JAZ1 to JAZ12), we carried out a targeted Y2H assay.

JAZ proteins were previously shown to interact with the F-box protein COI1 only in the presence of COR (an analog of JA-Ile) (Chini et al. 2007; Thines et al. 2007). We therefore, carried out Y2H analysis in the absence or presence of COR, with COI1 and JAZ9 as controls. Notably, SKIP31 interacted with JAZ6, JAZ10, and JAZ11 regardless of the presence of COR in the medium, but showed no clear interaction with other JAZs (Fig. 3A). COI1 was unable to interact with JAZ9 in the absence of COR (Fig. 3B). We conclude that, unlike COI1, the interaction between SKIP31 and JAZ proteins is COR-independent.

To confirm these interactions in plant cells, we conducted a BiFC transient assay in *N. benthamiana* leaves. We detected fluorescence from reconstituted yellow fluorescence protein (YFP) in the nucleus when we co-infiltrated the construct *nYFP-SKIP31* with *JAZ6-cYFP*, *JAZ10-cYFP*, and *JAZ11-cYFP* constructs. However, we observed no clear interaction between SKIP31 and JAZ1 or when we co-infiltrated the empty vector control (Fig. 3C).

To confirm the physical interaction of SKIP31 with JAZ proteins in vitro, we performed a pull-down assay with recombinant purified proteins. We incubated maltose binding protein (MBP)-tagged SKIP31 with His-tagged JAZ6 or JAZ11, using MBP as control. Following pull-down with amylose resin, we detected JAZ6 and JAZ11 in the reactions containing MBP-SKIP31, but not with MBP alone (Fig. 3D). Taken together, Y2H, BIFC, and in vitro pull-down assays confirm the physical interaction of SKIP31 with JAZ6 and JAZ11 in a COR-independent manner.

To delineate the precise domains in SKIP31, JAZ6, and JAZ11 responsible for their interaction, we tested different truncated versions of JAZ and SKIP31 for Y2H assays (Fig. 4, A to C; left panels). We determined that the deletion of the N terminus of SKIP31 containing the F-box domain does not prevent the interaction of SKIP31 with JAZ proteins, while the loss of the C terminus of SKIP31 disrupted the interaction (Fig. 4A; right panels). Similarly, we established that the Jas domain of JAZ6 and JAZ11 is essential for their interaction with SKIP31 (Fig. 4, B and C; right panels). Altogether, our data suggest that the Jas domains of JAZ6 and JAZ11 and the C terminus of SKIP31 mediate their interactions.

SKIP31 directs ubiquitination of JAZ proteins and their degradation via the 26S proteasomeTo check whether the SKIP31 interaction with JAZ proteins directs the ubiquitination of the JAZ proteins, we conducted an in vitro ubiquitination assay using purified recombinant proteins (MBP-SKIP31 and JAZ1-His, JAZ6-His, JAZ11-His) (Supplemental Fig. S11, A to C). To this end, we incubated purified JAZ6-His, JAZ11-His, or JAZ1-His (as a negative control) with a ubiquitin-activating enzyme (E1), a Ubiquitin-conjugating Enzyme H5a (E2-UbcH5b, human), ubiquitin (Ub), ATP/Mg^{2+} for 1 h at 30 °C, before subjecting the reactions to immunoblot analysis with antiubiquitin and anti-His antibodies. In parallel, we incubated each tested JAZ without ATP/Mg^{2+} and with or without SKIP31. We detected a characteristic ubiquitination pattern for JAZ6-His and JAZ11-His in the presence of SKIP31, but not for JAZ1 as determined with the anti-Ub antibody (Fig. 5A) and with the anti-His antibody (Supplemental Fig. S12), with no ubiquitination ladder in control reactions.

To determine whether SKIP31-mediated ubiquitination leads to the degradation of JAZ proteins, we conducted a cell-free degradation assay by incubating recombinant JAZ6-His and JAZ11-His with crude protein extracted from the indicated genotypes alone or with the proteasome inhibitor MG132. In our experiments, we used mutants in *COI1* (*coi1-16* and *coi1-1*) to rule out JAZ protein degradation via COI1. An immunoblot analysis showed that in the presence of MBP-SKIP31 and *coi1-16* protein extracts, the level of His-tagged JAZ proteins decrease gradually with longer incubation time in the absence of MG132, with only a trace amount of JAZ6-His and JAZ11-His remaining after 120 min incubation (Fig. 5, B and C). We obtained similar results when each tested JAZ-His was incubated with crude protein extracts from either WT or *coi1-1* (a null allele) (Fig. 5, D and E; Supplemental Fig. S13, A and B). However, we detected no 26S proteasomal degradation when JAZ1 (as a negative control) was incubated with *coi1-1* protein extracts (Supplemental Fig. S13C).

By contrast, when recombinant JAZ-His proteins were incubated with proteins extracts from a *SKIP31*-RNAi line and without added MBP-SKIP31, we observed no significant change in the abundance of recombinant JAZ6-His or JAZ11-His (Fig. 5, F and G). This finding confirmed the involvement of SKIP31 in the degradation of these JAZ proteins. In parallel, we treated similar reactions with MG132,

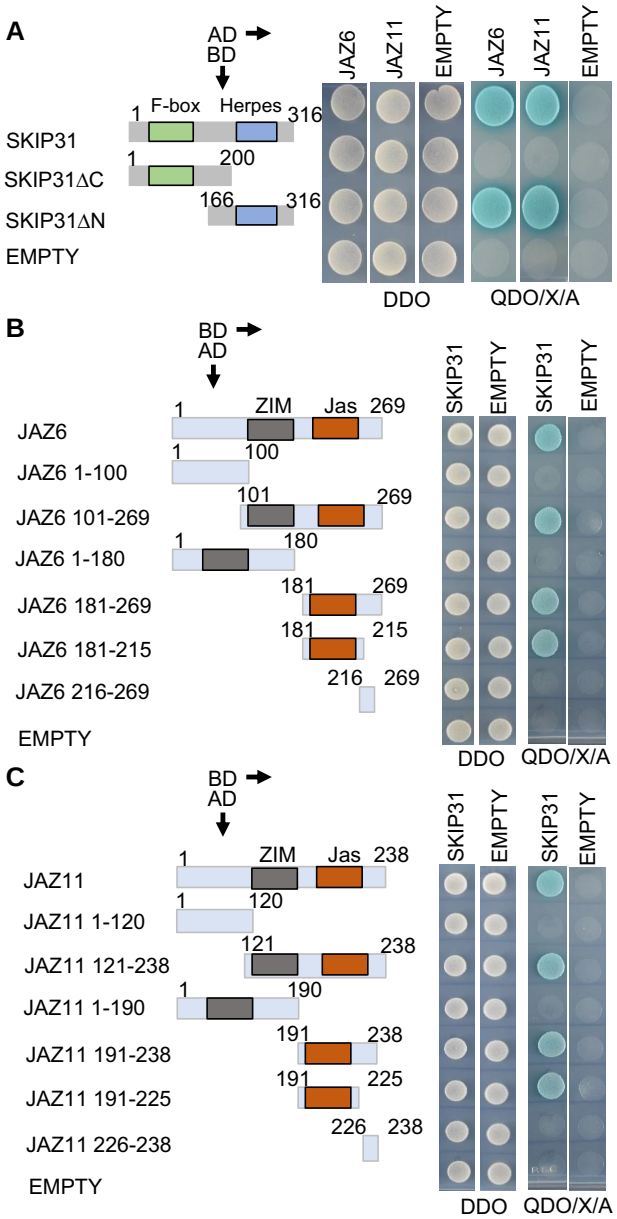

Figure 4. The C terminus of SKIP31 is important for its interaction with the jas domain of JAZs. **A)** The C-terminal part of SKIP31 is important for its interaction with JAZ proteins. Left, diagram of full-length and truncated SKIP31 variants lacking the F-box domain (SKIP31ΔC) or the Herpes domain (SKIP31ΔN) used in Y2H. Right, yeast Y2H gold colonies cotransformed with the indicated combinations of plasmids, spotted onto QDO medium lacking leucine, tryptophan, histidine, and adenine and containing with X-α-gal/aureobasidin agar (QDO/X/A) after 3 d of growth. **B and C)** The jas motif is crucial for interaction with SKIP31. Left, diagram of full-length and truncated variants of JAZ6 and JAZ11. Right, yeast Y2H gold cells colonies cotransformed with the indicated plasmids, spotted onto DDO medium lacking leucine and tryptophan; QDO medium lacking leucine, tryptophan, histidine, and adenine and containing with QDO/X/A after 3 d of growth. The empty vectors pDEST-GBKT7-(BD) and pDEST GADT7(AD) were used as negative controls.

resulting in unaltered levels of the indicated His-tagged JAZ proteins (Fig. 5, B to G; Supplemental Fig. S13). These findings validate SKIP31 as mediating the ubiquitination and proteasomal degradation of JAZ6 and JAZ11.

JAZΔjas-OE lines mimic the phenotype of *SKIP31*-RNAi lines

We next asked whether SKIP31 exerted its role in seed maturation and germination vigor by targeting JAZ proteins for proteasomal degradation in a JA-Ile independent manner. Accordingly, we generated OE lines for full-length *JAZ6* (*JAZ6*-FLOE) and *JAZ11* (*JAZ11*-FLOE) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. We confirmed increased transcript accumulation of *JAZ6* and *JAZ11* in their respective OE lines (Supplemental Fig. S14A). When we tested their germination patterns, we did not find any alteration in their germination ability compared to WT (Supplemental Fig. S14B), perhaps because these JAZ proteins are being degraded by SKIP31 in planta. We therefore generated another set of OE lines for JAZ6 and JAZ11 with their Jas deleted, yielding *JAZ6Δjas*-OE and *JAZ11Δjas*-OE, as the jas domain was found to be essential for the interaction with SKIP31. We confirmed the OE of *JAZ6Δjas* and *JAZ11Δjas* in their respective transgenic lines via RT-qPCR analysis (Supplemental Fig. S15, A and B). Phenotypic analyses revealed that like *skip31* mutants and *SKIP31*-RNAi, 4-wk-old *JAZ6Δjas* (*6OE1* and *6OE2*) and *JAZ11Δjas* (*11OE1* and *11OE2*) lines show a drastic drop in their germination rates (Fig. 6A; Supplemental Fig. S16A) and viability, as indicated by the lower accumulation of formazan and red coloration upon tetrazolium staining, relative to WT (Fig. 6B; Supplemental Fig. S16B). Additionally, like seeds from the *skip31*-RNAi line, the *JAZΔjas*-OE lines showed defects in the late seed developmental stages (16 to 21 DAF) of seed maturation, germination potential, and seed morphology (Fig. 6C; Supplemental Fig. S17, A to D). We also analyzed the germination potential of 0W to 8W postharvested seeds. As with seeds from the *skip31* mutant and *SKIP31*-RNAi lines, seeds from *JAZ6Δjas*-OE and *JAZ11Δjas*-OE lines showed significantly lower germination upon maturation and during storage compared to WT (Fig. 6D). By contrast, the mutants *jaz6* and *jaz11* showed no alteration in germination potential, viability, or seed morphology compared to WT (Fig. 6, A to D).

To check if the downregulation of COI1, which targets JAZ proteins in JA-Ile dependent manner, also influences seed germination, vigor, and/or viability like *SKIP31*-RNAi lines, we assessed germination and viability in the mutants *coi1-16* and *coi1-1*. Interestingly, the *coi1-16* and *coi1-1* mutants showed no defects in terms of germination ability, viability, or seed shape, with seeds similar to WT seeds (Fig. 6, A to D, Supplemental Fig. S18). Altogether, these data suggest that SKIP31 targets JAZ6 and JAZ11 in a JA-Ile-independent manner and positively regulates seed maturation, desiccation

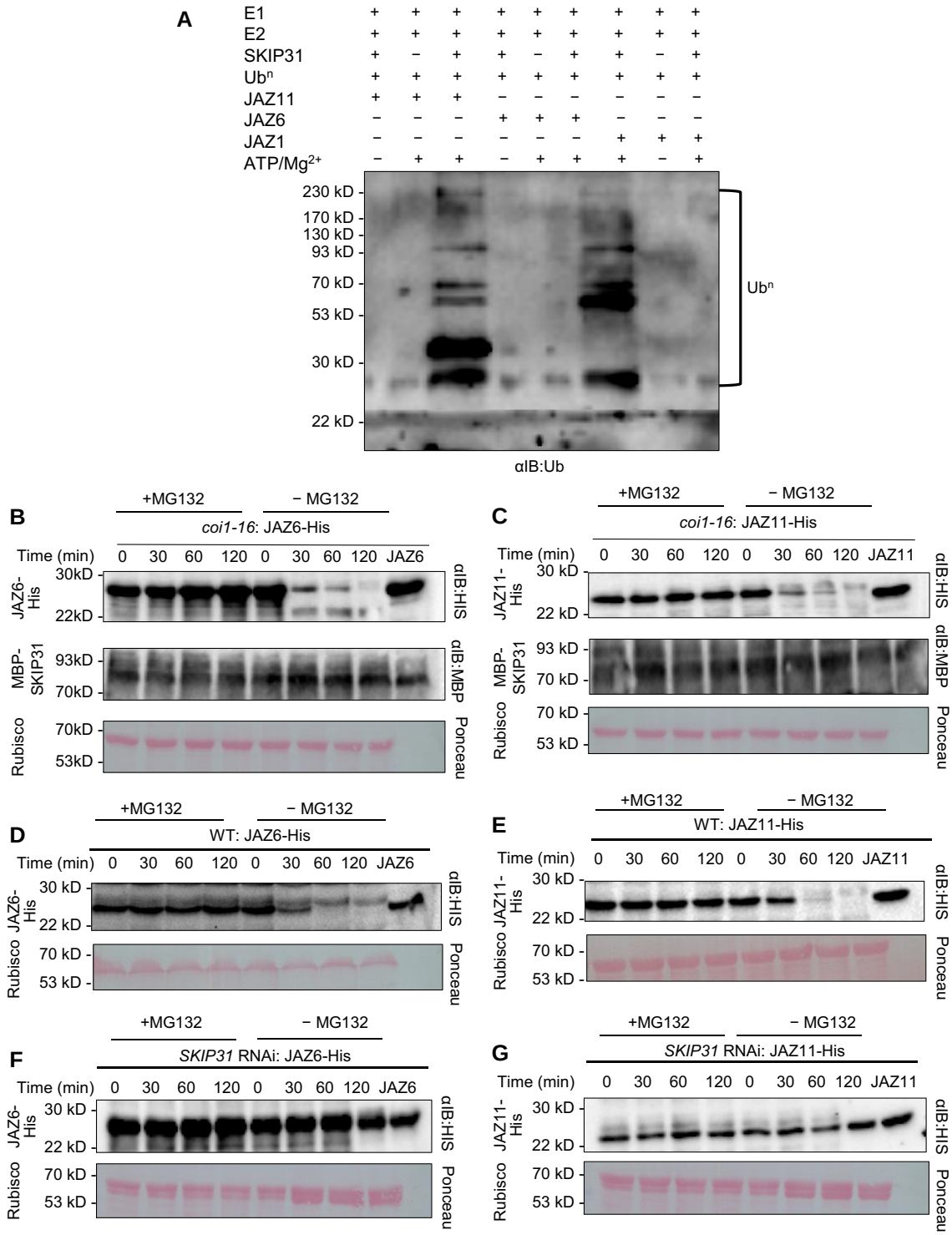

Figure 5. SKIP31 mediates the ubiquitination and degradation of JAZ6 and JAZ11 via the 26S proteasome. **A)** In vitro ubiquitination assay showing that SKIP31 mediates the ubiquitination of JAZ6 and JAZ11, but not JAZ1. The reactions were separated by SDS-PAGE and probed with an anti-ubiquitin (αIB-Ub) antibody. Each reaction contained an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme (UbcH5a), ubiquitin (Ub^n), ATP/Mg^{2+}, recombinant MBP-SKIP31, and the indicated recombinant JAZ-His (JAZ6-His, JAZ11-His, JAZ1-His) and was incubated at 30 °C for 1 h. **B to G)** Cell-free degradation assays for recombinant JAZ6-His **B, D, F)** or JAZ11-His **C, E, G)** at 30 °C for the indicated time period (in min) with or without MG132. Crude protein was extracted from ∼80 μg of seedling tissues from the indicated genotype and used in the reactions. The reactions were stopped by adding 5× SDS sample buffer and were processed for immunoblotting with anti-His and anti-MBP antibodies. Ponceau S staining of Rubisco large subunit was used as loading control.

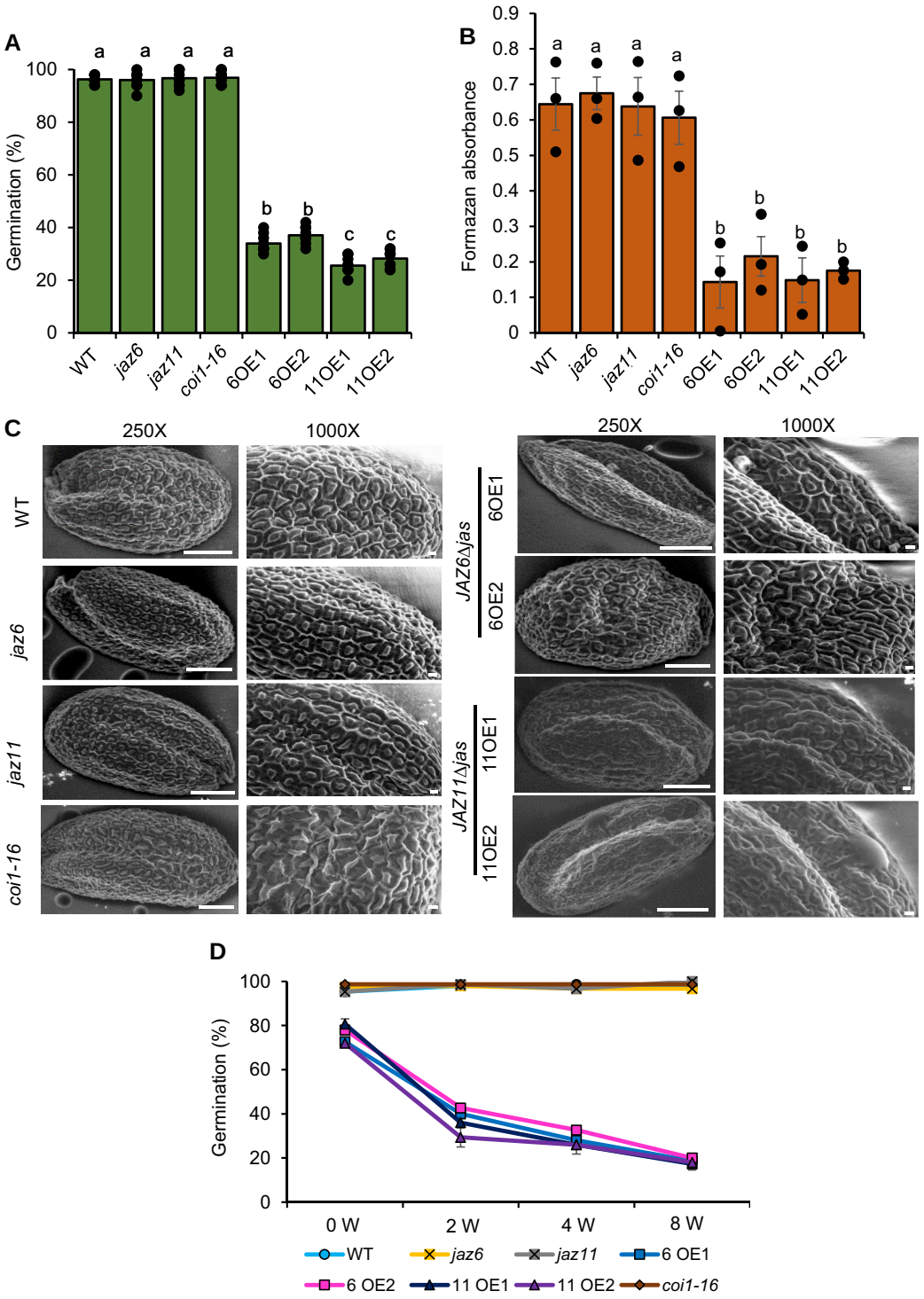

Figure 6. *JAZΔjas*-OE lines mimic the phenotype of *SKIP31*-RNAi lines. For all seed germination rate assays, mean and significance were calculated from 3 biological replicates grown and harvested at 3 different times. For each biological replicate, all genotypes were grown in identical conditions and harvested at the same time, and seeds were pooled from at least 20 independent plants and each seed batch was tested at least 3 times as technical replicates. The value of each biological replicate was the average calculated from 3 technical replicates (50 seeds per technical replicate). Data from 3 biological replicates were analyzed by ANOVA. Different lowercase letters indicate significant differences ($\alpha = 0.01$, as determined by ANOVA). **A)** Seed germination rates (4-wk-old seeds) from WT, *jaz6*, *jaz11*, *coi1-16*, *JAZ6Δjas* (6OE1 and 6OE2), and *JAZ11Δjas* OE lines (11OE1 and 11OE2) 7 d after stratification on water-agar medium. **B)** Formazan absorbance upon tetrazolium staining of 4-wk-old seeds from WT, *jaz6*, *jaz11*, *coi1-16*, *JAZ6Δjas* OE lines, and *JAZ11Δjas* OE lines. Seeds were incubated for 24 h in tetrazolium red at 28 °C, and formazan production was assayed by measuring the absorbance at 492 nm of 100 crushed seeds in 95% (v/v) ethanol. Values are means ± SE from 3 biological replicates. Different

(continued)

tolerance, and seed vigor in Arabidopsis. Furthermore, our data also indicate that the COI1-mediated pathway may not be directly involved in seed maturation and vigor.

JAZ proteins interact with ABI5 and downregulate the expression of target genes important for seed maturation, desiccation tolerance, and seed coat development directly or indirectly. JAZ proteins have been shown to modulate seed germination by interacting and repressing the transcriptional activity of ABI3 and ABI5 (Ju et al. 2019; Pan et al. 2020; Varshney and Majee 2021). In this study, we tested the interaction of JAZ6 and JAZ11 with ABI3 and ABI5 through BIFC. Indeed, we detected an interaction between JAZ6 or JAZ11 and ABI5, but not with ABI3 (Supplemental Fig. S19, A and B). The ABI3, ABI4, and ABI5-mediated signaling networks play a key role in seed maturation, desiccation tolerance, and seed vigor by activating a large subset of genes that are involved in several mechanisms necessary for achieving these seed traits. To investigate whether the SKIP31–JAZ–ABI5 module modulates the expression of downstream genes that are important for seed maturation and desiccation tolerance, we checked the expression of various ABI5 target genes and other marker genes of seed maturation such as *RD29A* (*RESPONSIVE TO DESICCATION 29A*), *RD29B*, *DREB2A* (*DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A*), *RAB18* (*RESPONSIVE TO ABA 18*) (drought and ABA-responsive genes); *EM1* (*LATE EMBRYOGENESIS ABUNDANT 1* [*LEA1*]), *EM6*; and the TFs ABI3, ABI4, and ABI5 in all indicated genotypes at the S5, S6, and S7 stages of seed development. Interestingly, the expression of most genes showed no significant changes at the S5 stage in *SKIP31*-RNAi and *JAZΔjas*-OE seeds compared to WT seeds (Fig. 7A). By contrast, all these genes were significantly downregulated in mature seeds for *SKIP31*-RNAi and the *JAZΔjas*-OE lines compared to WT seeds at the S6 and S7 stages., while *SKIP31*-OE seeds showed slightly increased or similar expression levels relative to WT (Fig. 7, B and C; Supplemental Fig. S20). These results suggest the role of the SKIP31–JAZ module during the late seed maturation stages, possibly affecting maturation drying, germination potential upon maturity, and ultimately overall seed vigor and viability.

Next, to check whether the SKIP31–JAZ–ABI5 module influences phytohormone levels, we quantified ABA, JA-Ile, and SA in seeds for WT, representative *SKIP31*-OE and *SKIP31*-RNAi lines. We determined that *SKIP31*-RNAi seeds, which exhibit altered seed traits, accumulate less ABA than WT and *SKIP31*-OE seeds (Fig. 7D). However, the levels of JA-Ile and SA were similar across all genotypes (Fig. 7, E and F), further supporting the lack of involvement of the JA-Ile pathway in the SKIP31–JAZ module. These results also indicate that SKIP31 regulates ABA accumulation in seeds; to support this hypothesis, we checked the expression of ABA biosynthetic genes (*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* [*NCED3*] and *NCED6*), which showed lower transcript levels in seeds from the *SKIP31*-RNAi line compared to seeds from WT and *SKIP31*-OE (Fig. 7, G and H; Supplemental Fig. S20).

Overall, the decreased expression levels of several target genes, as well lower ABA accumulation, which is important for seed maturation and thus seed vigor and viability, indicate that downregulation of *SKIP31* results in overaccumulation of its target proteins (JAZ6, JAZ11), which in turn repress ABI5 and possibly other TFs and regulators. ABI5 and other regulators subsequently negatively regulate the expression of the above-discussed genes, directly or indirectly, in *SKIP31*-RNAi and *JAZΔjas*-OE lines.

The SKIP31–JAZ module affects seed reserve accumulation

Since *SKIP31*-RNAi and *JAZΔjas*-OE seeds were shrunken and distorted, with compromised seed maturation, vigor, and/or viability, we were also interested in investigating seed filling, particularly the accumulation of storage compounds like seed storage proteins (SSPs), sucrose, and Raffinose Family of Oligosaccharides, which are all an integral part of seed maturation and are also largely regulated by ABA signaling and the ABI3-, ABI4-, and ABI5-mediated networks (Kroj et al. 2003; Kagaya et al. 2005; Gutierrez et al. 2007; Verdier and Thompson 2008; González-Morales et al. 2016). We thus measured the contents of carbohydrates (sucrose and starch), total soluble seed proteins, and total lipids in mature seeds of the indicated genotypes. We observed a significant variation in the total amount of soluble sugars, particularly sucrose, and total soluble proteins containing SSPs among *SKIP31*-RNAi and *JAZΔjas*-OE lines compared to WT (Fig. 8, A and B). However, the total starch and lipid contents were similar in all tested genotypes (Fig. 8, C and D).

Simultaneously, we also measured the contents of other protective metabolites through untargeted gas chromatography-MS (GC-MS) analysis in the mature dry seeds of the representative lines for *SKIP31*-RNAi and *JAZΔjas*-OE. We observed compromised accumulation of myo-inositol (the precursor of galactinol) and galactinol in all indicated genotypes relative to WT (Fig. 8, E and F). The role of galactinol and myo-inositol has also been shown to be important in desiccation tolerance and to be influenced by ABA (Salvi et al. 2016, 2020, 2022).

As we detected lower accumulation of sucrose, galactinol, and myo-inositol, we investigated the expression levels of the

(**Figure 6.** Continued)
lowercase letters indicate significant differences ($\alpha = 0.01$, as determined by ANOVA). **C)** Representative SEM micrographs of mature dry seeds from the indicated Arabidopsis genotypes. Scale bars, 100 μm (250×), 10 μm (1,000×). **D)** Seed germination rates scored at 7 d after stratification on water-agar medium for WT, *jaz6*, *jaz11*, *coi1-16*, *JAZ6Δjas* OE lines, and *JAZ11Δjas* OE lines from 0W to 8W postharvested seeds. Values are means ± SE from 3 biological replicates. xW, x-wk-old post harvested seeds.

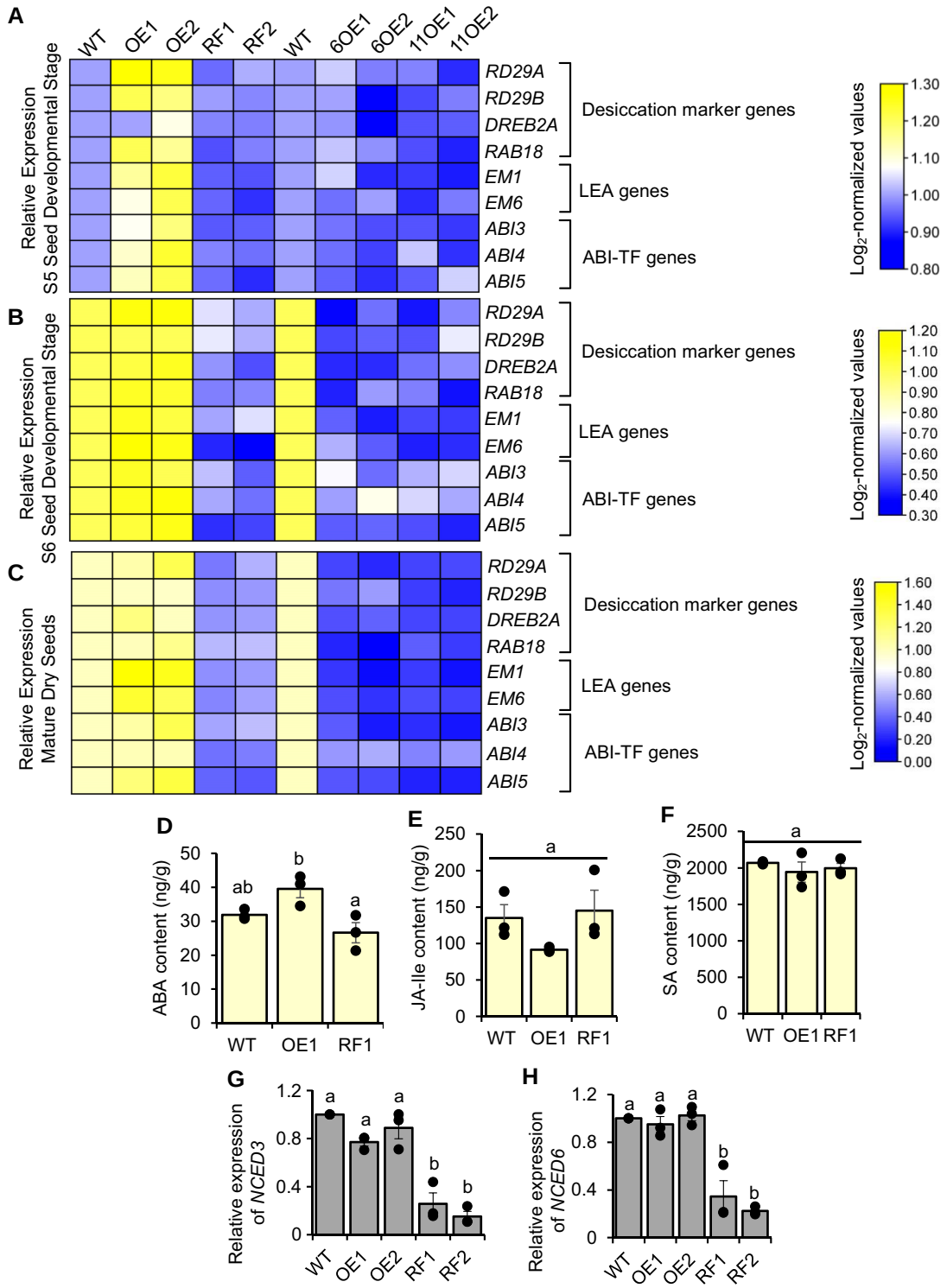

Figure 7. *SKIP31*-RNAi and *JAZΔjas*-OE lines exhibit differential expression of several genes important for seed maturation and ABA response. **A to C)** Heatmap showing the differential expression of genes important for seed maturation and ABA response in the indicated genotypes. Relative expression at the S5 seed developmental stage (13 to 15 DAF) **A)**, S6 seed developmental stage (16 to 18 DAF) **B)**, and mature dry seeds **C)** based on RT-qPCR data. Expression values were determined by the ΔΔCT method and normalized to *ACTIN2* expression. Values are Log2-normalized means of 3 biological replicates. The heatmap was generated with TB tools; values are given in Supplemental Data Set 2. Blue indicates low expression; yellow indicates high expression. **D to F)** Contents of the phytohormones ABA **D)**; JA-Ile **E)**; and SA **F)** in mature dry seeds of WT, one representative *SKIP31*-OE line (OE1) and one representative *SKIP31*-RNAi line (RF1). All concentrations are given as ng/g (SW). Values are means ± SE of 3 replicates. Different lowercase letters indicate significant differences ($\alpha = 0.01$, as determined by ANOVA). **G and H)** RT-qPCR analysis of relative

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genes respectively involved in sucrose and galactinol metabolism. For instance, *GALACTINOL SYNTHASE 1* (*GolS1*) and *SUCROSE SYNTHASE 3* (*SUS3*) have been suggested to contribute to galactinol and sucrose metabolism, respectively, in the late stages of seed development and have also been reported to be influenced by ABA and desiccation (González-Morales et al. 2016; Salvi et al. 2018, 2016; Zhang et al. 2019). Our RT-qPCR analysis suggested that *SKIP31*-RNAi and *JAZΔjas*-OE lines express *GolS1* and *SUS3* to lower levels than WT seeds (Fig. 8, G and H). Previously, a reduction in the accumulation of oligosaccharides, and lower transcript levels of oligosaccharide biosynthesis genes encoding key enzymes were shown to strongly repress desiccation-sensitive genotypes (González-Morales et al. 2016). Similarly, an analysis of the expression of genes encoding SSPs like *CRUCIFERIN3* (*CRU3*; precursor of globular 12S proteins) and *2S2* (precursor of albumin 2S proteins) in the indicated representative transgenic lines revealed their significantly lower expression in *SKIP31*-RNAi and *JAZΔjas*-OE lines (Fig. 8, I and J). Interestingly, the expression levels of *CRU3* and *2S2* were significantly downregulated at the S5 seed developmental stage in *SKIP31*-RNAi and *JAZΔjas*-OE seeds compared to WT seeds, while seeds from the *SKIP31*-OE line showed no significant differences in the expression of these genes (Supplemental Fig. S21).

These data confirm that the SKIP31–JAZ module indeed facilitates the expression and function of genes and their encoded products required for seed maturation and consequent seed vigor, directly or indirectly.

ABI5 positively regulates *SKIP31* expression, while JAZ proteins repress ABI5-mediated transactivation of *SKIP31* expression

Our expression analysis revealed that *SKIP31* transcript levels are highly upregulated during late maturation and in the presence of ABA and dehydration stress. To understand the regulation of *SKIP31* expression, particularly in seeds, we analyzed ~1.6 kb of regulatory sequences in the *SKIP31* promoter upstream of the ATG. Interestingly, we noticed 2 abscisic acid (ABA)–responsive elements (ABREs), ACGT located at positions 305 and 257 bp upstream from the ATG (Fig. 9A; Supplemental Fig. S22). ABREs are known to be targeted by ABI5 (Carles et al. 2002; Ju et al. 2019). Therefore, to examine whether ABI5is involved in the regulation of *SKIP31* expression in seeds, we performed a dual luciferase (LUC) assay, in *N. benthamiana* leaves, using a *proSKIP31:LUC* reporter construct (Fig. 9B) and *35S:ABI5-YFP* as an effector construct (Fig. 9C). As shown in Fig. 9D, the relative LUC activity derived from the *SKIP31* promoter was upregulated when *proSKIP31:LUC* was coinfiltrated with *35S:ABI5-YFP*, but not with *35S:YFP*. Considering the fact that JAZ proteins physically interact with ABI5, and repress the transcriptional activity of ABI5, we asked whether JAZ also repressed ABI5-mediated expression of *SKIP31*. To this end, we generated effector constructs for JAZ6 (*35S:JAZ6-YFP*) and JAZ11 (*35S:JAZ11-YFP*) (Fig. 9C). Our data revealed that the intensity of LUC activity significantly decreases when *JAZ* and *ABI5* are co-expressed compared to *JAZ* or *ABI5* being expressed with the *proSKIP31:LUC* reporter, indicating that JAZ proteins disrupt ABI5-activated *LUC* expression (Fig. 9D). These results suggest that ABI5 positively regulates *SKIP31* expression in seeds, but that ABI5 activity is repressed by JAZ proteins, raising the possibility of a positive feedback regulation of ABI5-mediated expression of *SKIP31* and JAZ degradation.

To corroborate the involvement of ABI5 in the SKIP31–JAZ module, we tested the expression levels of chosen genes in seeds of the *abi5-1* mutant and its WT Ws-2 at the S5 stage and in dry mature seeds. Interestingly, the expression of the *LEA* genes *EM1* and *EM6*, *DREB2A*, *SKIP31*, and *2S2* were significantly downregulated in *abi5-1* mutant seeds at the S5 and dry seeds stages, while other genes like *ABI3*, *ABI4*, and *CRU3* showed little or no changes in their expression at either stage (Fig. 9, E and F; Supplemental Fig. S23).

Furthermore, to validate the role of ABI5 in this module, we generated *ABI5*-OE lines in the *SKIP31*-RNAi background (Supplemental Fig. 24, A and B) and checked their germination potential. Interestingly, the OE of *ABI5* partially rescued the low germination rate of the *SKIP31*-RNAi lines, reaching about ~80% to 85% germination compared to WT (Supplemental Fig. S24C). Although *SKIP31* transcript levels remained low in these lines, possibly due to active RNAi suppression of *SKIP31* transcripts, *ABI5* was overexpressed in *ABI5*-OE *SKIP31*-RNAi lines, possibly partially restoring the germination rate of the *SKIP31*-RNAi line. However, *ABI5*-OE in the WT background (Supplemental Fig. S24D) behaved like WT in terms of germination potential, in accordance with previous studies (Chen et al. 2008; Lopez-Molina et al. 2001; Yang et al. 2021) (Supplemental Fig. S24E). We also tested the dormancy level of freshly harvested seeds for the *skip31* mutant and *SKIP31*-RNAi lines; like the *abi5-1* mutant, we did not observe any significant changes in the dormancy pattern of the *skip31* mutant or *SKIP31*-RNAi lines compared to WT seeds (Supplemental Fig. S25).

Altogether, this study supports the partial involvement of ABI5 in the SKIP31–JAZ module in the seed maturation and viability phenotypes of the *skip31* mutant and *SKIP31*-RNAi lines, and thus raises the intriguing possibility of the involvement of other TFs and/or regulatory proteins in this module.

(**Figure 7.** Continued)
transcript levels for *NCED3* **G**), *NCED6* **H**) in seeds from WT, *SKIP31*-OE1, and *SKIP31*-RNAi 1 (RF1). Expression values were determined by the ΔΔCT method and normalized to *ACTIN2* expression. Values are means ± SE of 3 biological replicates. Different lowercase letters indicate significant differences ($\alpha = 0.01$, as determined by ANOVA). SW, seed weight.

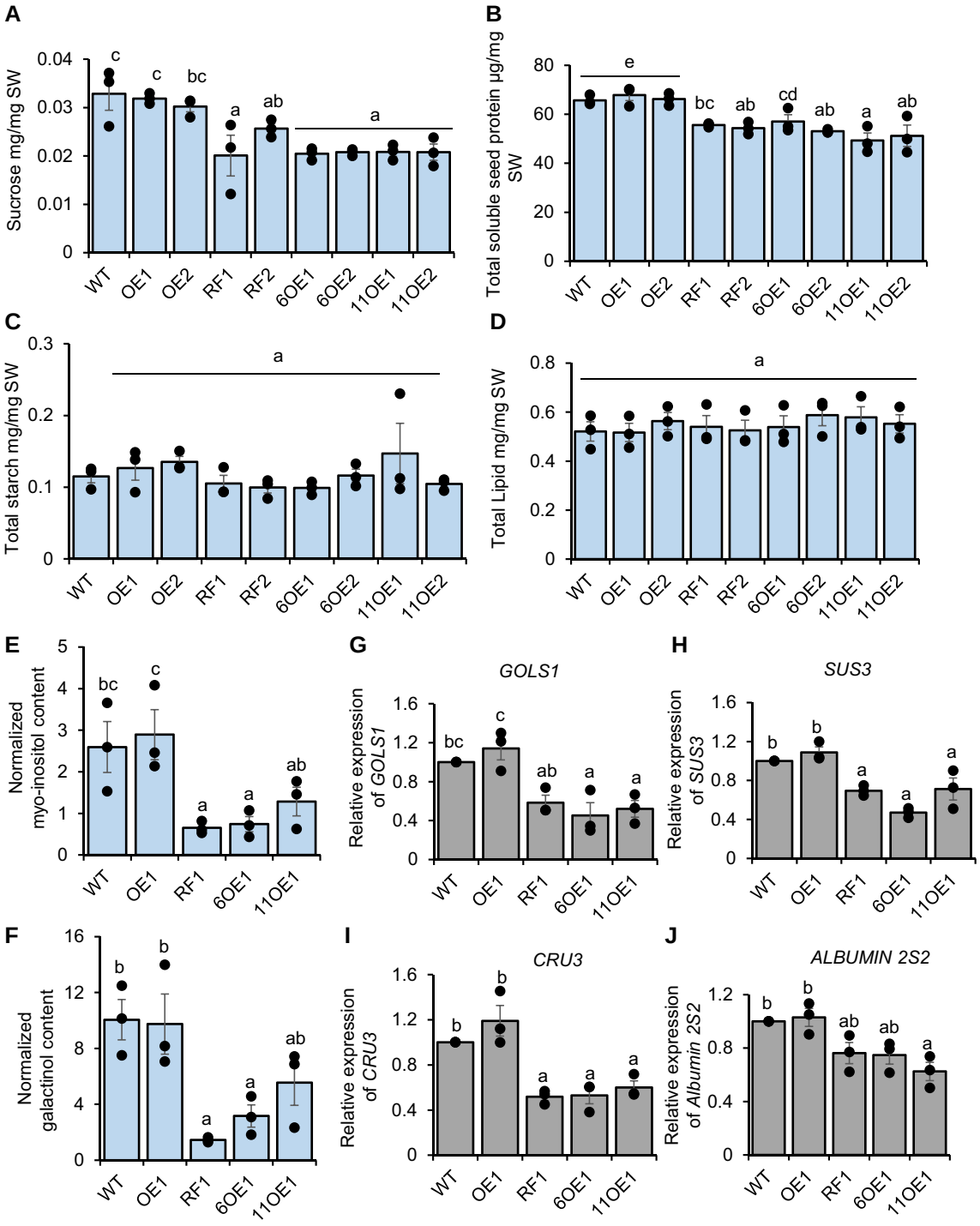

Figure 8. The SKIP31–JAZ module affects the accumulation of seed reserves and the expression of associated genes in Arabidopsis seeds. **A to D)** Contents of carbohydrates (sucrose and starch), SSPs, and lipid composition in mature dry seeds of the indicated Arabidopsis genotypes. **A)** Total sucrose content. **B)** Total soluble seed protein content. **C)** Total starch content. **D)** Total lipid content. Values are means ± SE of 3 biological replicates. Different lowercase letters indicate significant differences ($\alpha = 0.05$). **E, F)** Relative content of galactinol **E)** and myo-inositol **F)** in the mature dry seeds of the indicated Arabidopsis genotypes by GC-MS, using adinitol (20 mg/mL) as internal standard. Values are means ± SE of 3 biological replicates. Different lowercase letters indicate significant differences ($\alpha = 0.05$). **G to J)**, RT-qPCR analysis of the relative transcript levels of *GolS1* **G)**; *SUS3* **H)**; *CRU3* **I)**; and *ALBUMIN2S2* **J)** in the mature dry seeds of indicated Arabidopsis genotypes. Expression values were determined by the ΔΔCT method and normalized to *ACTIN2* expression. Values are means ± of 3 replicates. Different lowercase letters indicate significant differences ($\alpha = 0.01$, as determined by ANOVA). SW, seed weight.

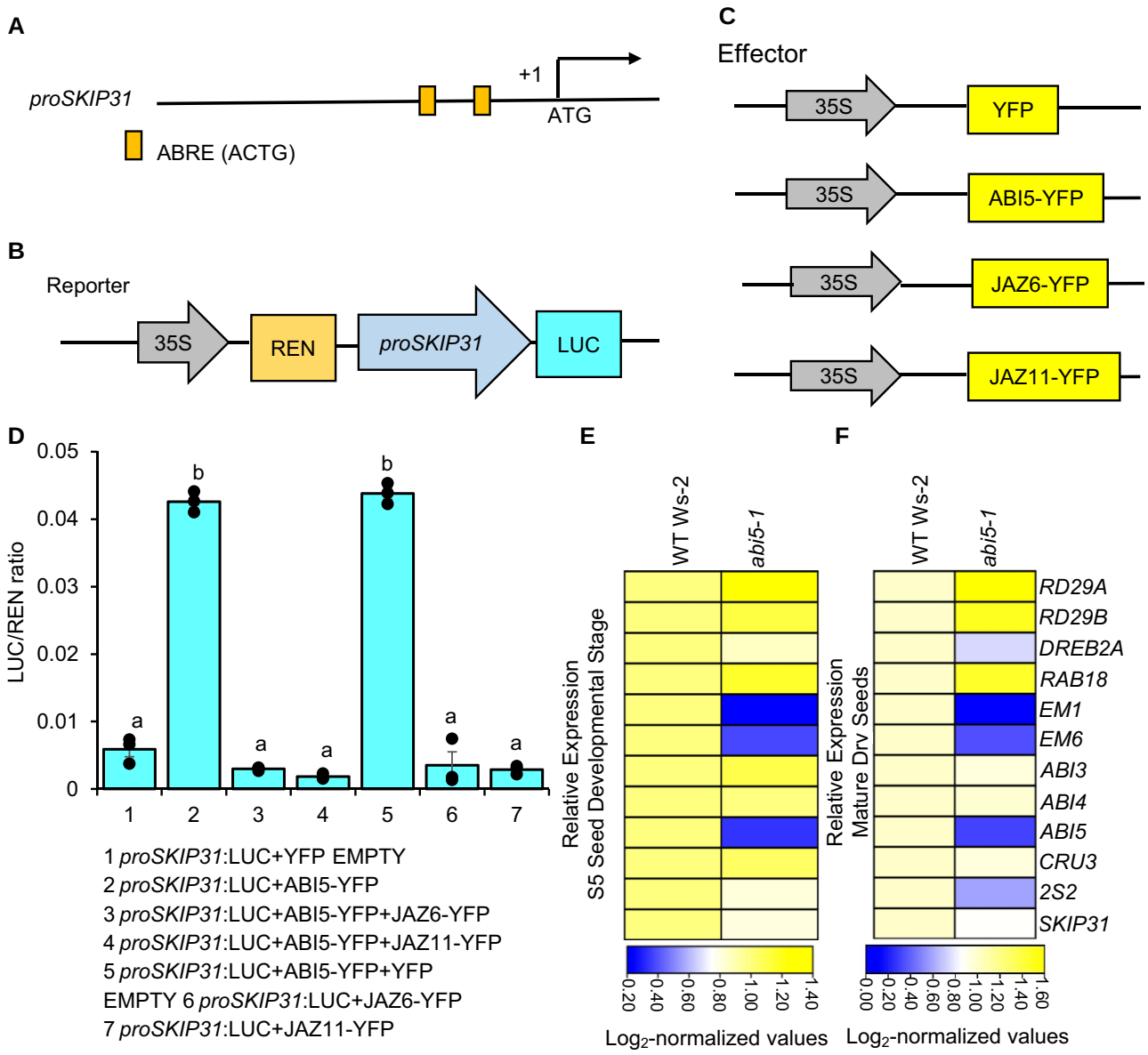

Figure 9. ABI5 positively regulates *SKIP31* expression, while JAZ proteins repress ABI5-mediated transactivation of *SKIP31*. **A)** Diagram of the *SKIP31* promoter showing the putative ABA-response elements (ABREs) (rectanglular boxes). The translational start site (ATG) is shown as +1. **B, C)** Diagram of the reporter **B)** and effector **C)** constructs used in the transient transactivation assays. **D)** Relative LUC/REN activity in *Nicotiana benthamiana* leaves coinfiltrated with the reporter construct pro*SKIP31:LUC* and the indicated effector constructs. Renilla luciferase (REN) from a *35S:REN* construct was used for normalization. Values are means ± SE of 3 biological repeats. Different lowercase letters indicate significant differences ($\alpha = 0.01$, as determined by ANOVA). **E, F)** Heatmap showing the relative expression of genes important for seed maturation and ABA response in the *abi5-1* mutant and its WT Ws-2. Relative expression analysis at the S5 seed developmental stage (13 to 15 DAF) **E)**, or in mature dry seeds **F)** based on RT-qPCR data. Expression values were determined by the ΔΔCT method and normalized to *ACTIN2* expression. Values are means of 3 biological replicates and were Log2-normalized to generate the heatmap with TB Tools; the values are given in Supplemental Data Set 2. Blue indicates low expression; yellow indicates high expression.

Discussion

Seed maturation, particularly maturation drying, acquisition of desiccation tolerance, seed storage reserve accumulation, allows seeds to extend their viability and germination potential in a desiccated state. Seed maturation is a highly complex and coordinated molecular event that is largely mediated by the phytohormone ABA and its downstream signaling pathway. The ABA signaling pathway mainly involves the ABA receptors PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-LIKE/REGULATORY COMPONENT OF ABA RECEPTOR, the type 2C protein phosphatases ABI1 and ABI2, SNF1-RELATED PROTEIN KINASE 2s (Ma et al. 2009; Zhao et al. 2020), and different master regulators, such as the

TFs ABI3, ABI4, ABI5, FUS3, LEC1, and LEC2, that participate and activate several downstream genes and developmental programs, eventually exerting ABA responses (To et al. 2006; Gutierrez et al. 2007; Santos-Mendoza et al. 2008; González-Morales et al. 2016). The role of ABI3 and ABI4 in seed maturation has long been known. Several studies suggest that besides ABI3 and ABI4, ABI5 also participates in seed maturation and germination by modulating the expression of various target genes including *EM1* and *EM6* by directly binding to the ABRE present within their promoter regions (Carles et al. 2002; Ju et al. 2019). Previous studies also revealed that these ABA-related TFs exhibit extensive cross-regulation, and they encoding genes are often coexpressed and act synergistically while regulating ABA-controlled programs, like seed maturation, dormancy, germination, seed vigor, and viability (Kermode 2017; Ali et al. 2022). For instance, ABI4 acts synergistically with ABI5 in controlling seed germination and sugar sensitivity, and a subset of ABI4 target genes are also ABI5 targets (Bossi et al. 2009; Söderman et al. 2000; Reeves et al. 2011). Likewise, ABI3 is also a positive upstream regulator of ABI5 (Lopez-Molina et al. 2002). Therefore, disruption or modulation of the expression and function of one factor affects that of the other factor. Despite the identification of several positive and negative regulators of ABA signaling, how these regulators accurately and coordinately achieve differential ABA signaling during phase transition from seed development, and maturation to germination events remains obscure. Recently, the regulatory role of JAZ proteins in repressing ABA signaling during seed germination has been documented by interacting and repressing the activity of the TFs ABI3 and ABI5 (Ju et al. 2019; Pan et al. 2020; Varshney and Majee 2021). The present work reveals how this JAZ-mediated repression of ABA signaling and ABI5 activity is alleviated during seed maturation by an F-box protein (SKIP31) and provides new insight into ABA-mediated seed maturation, desiccation tolerance, and seed vigor. Our work demonstrates (i) the regulatory role of the F-box protein SKIP31 protein in seed maturation, seed vigor, and viability by targeting JAZ proteins; (ii) the participation of the SKIP31–JAZ module in regulating the activity of ABI5, through repression and derepression, which in turn modulate the expression of several genes involved in seed maturation, desiccation tolerance, and seed vigor; and (iii) ABI5-mediated regulation of *SKIP31* expression.

We show that, similar to various genes encoding proteins associated with seed maturation (Probert et al. 2007; Verdier et al. 2013; Righetti et al. 2015), *SKIP31* expression sharply increases at the late maturation phase, when the seed prepares for maturation drying and other associated events mediated by ABA signaling, and then declines during germination when ABA signaling is generally repressed (Fig. 1, D and E). This increased expression of *SKIP31* at the late maturation phase and in the presence of ABA (Fig. 1F) supports its participation in seed maturation. Subsequently, genetic analyses on *skip31* mutants, *SKIP31*-RNAi, complementation, and seed-specific OE lines demonstrated its regulatory role in seed maturation and consequently seed vigor and viability (Fig. 2). We show that SKIP31 physically interacts with the JAZ proteins, JAZ6, JAZ10, and JAZ11 in particular, which does not require COR, in contrast to the previously reported COI1-mediated interaction and degradation of JAZ proteins (Fig. 3). Our study also reveals that SKIP3–JAZ interactions are mediated by the C-terminal Jas domain of JAZ proteins (Fig. 4), which is also the site of COI1–JAZ interactions (Katsir et al. 2008; Melotto et al. 2008; Sheard et al. 2010). Our data further provide evidence that SKIP31 facilitates ubiquitination and subsequent degradation of indicated JAZ6 and JAZ11 (Fig. 5). JAZ proteins, which act as repressors, exhibit a high level of interaction and functional redundancy; however, distinct interaction specificities have also been reported (Pauwels et al. 2010, 2015; Hu et al. 2013; Jiang et al. 2014; Zhai et al. 2015).

In our study as well, we show that only 3 specific JAZ proteins (JAZ6, JAZ10, and JAZ11) interact with SKIP31. Our characterization of *JAZΔjas*-OE lines further support the notion that SKIP31 targets JAZ proteins for proteasomal degradation during seed maturation, as *JAZ6Δjas*-OE and *JAZ11Δjas*-OE lines show defects in seed morphology, maturation, desiccation tolerance, and viability similar to those of the *skip31* mutant and *SKIP31*-RNAi lines. We did not observe any defects in maturation, distorted phenotype, or germination in the case of *coi1* mutants Fig. 6), indicating that the COI1JAZ pathway may not have any involvement in seed maturation. This idea is in accordance with a recent study that showed that a mutation in *COI1* leads to decreased ABA signaling during seed germination but did not provide any evidence about its involvement in seed maturation (Pan et al. 2020). Thus, it would be interesting to determine how COI1 and SKIP31 are precisely regulated to selectively target JAZ proteins during developmental phase transitions in JA-Ile-dependent and -independent manners, respectively.

Further, in agreement with previous studies where JAZ proteins were shown to interact with ABI5 (Ju et al. 2019; Pan et al. 2020), in the *skip31* mutant, *SKIP31*-RNAi, and *JAZΔjas*-OE lines, a possible accumulation of JAZ proteins repress ABI5 function and downregulates the expression of genes that are important to seed maturation. Additionally, phytohormone analysis in all genotypes suggests that SKIP31 not only positively influences ABA signaling but also influences ABA biosynthesis in seeds (Fig. 7). Our study provides evidence that SKIP31 derepresses ABI5 activity by targeting JAZ6 and JAZ11 for degradation to promote ABA signaling during seed maturation. The lower expression of *LEA* genes, such as *EM1*, *EM6*, and storage deposition-associated genes, and ABA-related TF genes, which are essential for seed maturation including desiccation tolerance and seed viability, in *SKIP31*-RNAi lines and *JAZ6Δjas*-OE and *JAZ11Δjas*-OE lines further validate our findings. Interestingly, even though a role for ABI5 in regulating seed maturation and germination has been proposed, a defect

in seed dormancy, germination, and viability of freshly harvested seeds has not been reported in *abi5* mutants, possibly because the loss of ABI5 function in Arabidopsis may be compensated by the presence of partially redundant proteins (Finkelstein 1994; Finkelstein et al. 2008). Nonetheless, ABI5 was shown to be important for the reinduction of desiccation tolerance in germinating Arabidopsis seeds (Maia et al. 2014) and in seed maturation and longevity in legumes (Zinsmeister et al. 2016). A significant reduction in seed storage accumulation particularly sucrose and SSPs, and metabolites like galactinol and myo-inositol, accumulated during seed maturation largely regulated by ABA and ABI TF including ABI5 directly or indirectly, in *SKIP31* RNAi lines and *JAZ6Δjas*-OE and *JAZ11Δjas*-OE lines were also observed (Fig. 8). These metabolites are not only important for maintaining the shape and size of seeds but also positively influences seed vigor (Dekkers et al. 2015; Leprince and Buitink 2015; Righetti et al. 2015; Leprince et al. 2017). The nonoverlapping phenotypes of *skip31* and *abi5* mutants in terms of seed germination and viability upon seed maturity is possibly reflect the possibility that the SKIP31–JAZ module not only regulates ABI5 function but also other possible TFs and/or regulatory proteins important for seed maturation and desiccation tolerance (González-Morales et al. 2016). Furthermore, how LEC1, LEC2, and FUS3, coordinate with the SKIP31–JAZ–ABI5 module to regulate seed maturation events is also an interesting area to explore. Additionally, it remains to be examined whether SKIP31 can also influence JA-Ile-dependent regulation of the JAZ–MYC module in the JA signaling pathway. Our analysis regarding the regulation of *SKIP31* expression mediated by the ABI5–JAZ module also indicates a possible feedback regulation of SKIP31 (Fig. 9). Furthermore, partial restoration of the seed germination defect upon OE of *ABI5* in *SKIP31*-RNAi lines further validates the role of this SKIP31–JAZ–ABI5 module in seed maturation, and raises the intriguing possibility that other regulators are involved in this module (Supplemental Fig. S24C).

Altogether, our work provides evidence that SKIP31 targets the JAZ proteins JAZ6 and JAZ11 for proteasomal degradation, in a JA-Ile-independent manner, which in turn releases their inhibitory effect on ABI5 activity and possibly other seed maturation regulators, which are essential for seed maturation and consequently seed vigor (Fig. 10).

Material and methods

Plant materials and growth conditions

All genotypes used in this study were in the Arabidopsis (*A. thaliana*) accession Columbia-0 (Col-0), except *abi5-1*, which is in Ws. The T-DNA insertion lines for *SKIP31* (CS848440 and CS829396), *JAZ6* (CS370178), and *JAZ11* (SALK_095426C), *coi1-1* (CS9721), and *abi5-1* (in Ws) were obtained from the. The *coi1-16* mutant was obtained from Dr. Jyothilakshmi Vadassery's lab at NIPGR, New Delhi, India. *N. benthamiana* plants were used for subcellular localization, BiFC, and Dual-LUC assays. All plants were grown in growth chambers (22 ± 2 °C with a 16-h light/8-h dark photoperiod with a light intensity of 120 μmol/m^2/s, white fluorescent tube from Philips).

Generation of transgenic Arabidopsis lines

All OE lines were generated in Col-0 using suitable constructs. To generate seed-specific *SKIP31* OE lines, the *SKIP31* coding sequence was placed under the control of the seed-specific promoter *proNAPIN* in a modified pCAMBIA2301 vector. For the-RNAi construct, a unique sequence of 510 bp (439 to 948 bps) *of SKIP31* fragment were cloned in pENTR/D-TOPO, followed by LR recombination into the pK7GWIWG2 II-Red Root vector to generate the ihpRNAi cassette (Supplemental Fig. S4). For generating *proSKIP31:GUS* lines, a suitable ß-glucuronidase (*GUS*) promoter construct was made using Gateway cloning. Briefly, a ~1.6-kb region upstream of *SKIP31* was amplified from Col-0 genomic DNA and cloned upstream of *GUS* via Gateway cloning into the *GUS* vector pMDC164. For complementation studies, the *proNAPIN:SKIP31* construct was used to transform plants from the *skip31* mutant CS848440. Similarly, to generate full-length *JAZ6*, *JAZ11*, and Δjas-truncated versions of *JAZ6* and *JAZ11* (*JAZ6Δjas* and *JAZ11Δjas*)-OE lines, the full-length *JAZ6* and *JAZ11* coding sequences, as well as their Δjas-truncated variants (*JAZ6Δjas* and *JAZ11Δjas*) were cloned into the pCAMBIA1301 vector.

To overexpress *ABI5* in *SKIP31*-RNAi and Col-0, the *ABI5* coding sequence was placed under the control of the CaMV 35S promoter in the pEG101 vector. All constructs were transformed into Arabidopsis Col-0 plants using the Agrobacterium-mediated floral dip method (Clough and Bent 1998). Transgenic plants were selected based on the relevant antibiotic resistance, reporter gene expression, and RT-qPCR analysis. The primers used in the study are listed in Supplemental Data Set 1.

Analysis of mutant seeds

Transcript accumulation was quantified in seeds from the respective mutants by RT-qPCR. The primers used are listed in Supplemental Data Set 1.

Expression analyses and RT-qPCR

Total RNA was extracted from tissue samples (seedlings, leaves, and seeds) using TRIzol reagent as described by Kaur et al. (2013) with minor modifications. Fifty $\pm$ 5 mg seeds or indicated tissues were collected for RNA extraction. One microgram DNaseI treated RNA was reverse-transcribed using a verso cDNA synthesis kit (Thermo Scientific) using the manufacturer's protocol. qPCR was carried out as described by (Rao et al. 2018) using suitable primer pairs (Supplemental Data Set 1). Gene expression was normalized to the expression of *ACTIN* or At4g12590 or At4g34270 (Dekkers et al. 2012) as an endogenous control. The

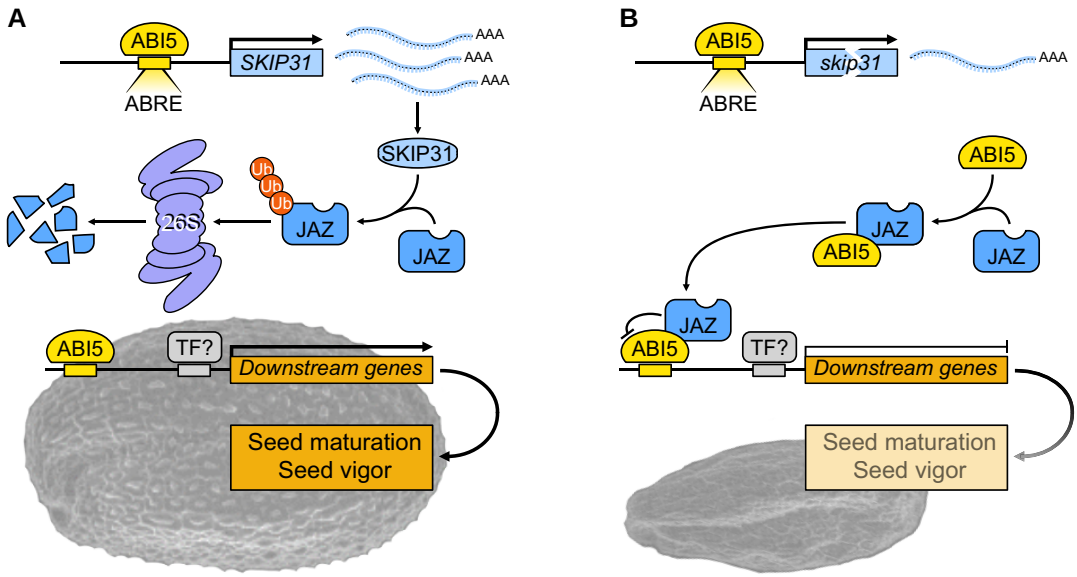

Figure 10. A working model describing the role of SKIP31 in the regulation of seed maturation and seed vigor by targeting JAZ proteins. *SKIP31* is predominantly expressed in seeds. SKIP31 interacts with JAZ proteins, particularly JAZ6 and JAZ11, and ubiquitinates them for degradation via the 26S proteasome. JAZ proteins suppress the activity of ABI5 and possibly other TFs, thus repressing the expression of downstream genes, directly or indirectly, that are required for proper seed maturation, and consequently seed vigor and viability. **A)** In WT, SKIP31 mediates the 26S proteasomal degradation of JAZ proteins, thus freeing ABI5 and possibly other TF(s), allowing the expression of downstream genes important for seed maturation and vigor. **B)** In *SKIP31*-RNAi seeds with lower *SKIP31* levels, JAZ proteins accumulate, which represses the transcriptional activity of ABI5 and possibly other TF(s) and inhibits the expression of downstream genes, ultimately affecting seed maturation, vigor, and viability. Solid arrows represent direct regulation; dashed arrows represent indirect or unknown regulation. (?) represents the unknown TF(s).

$2^{-\Delta\Delta CT}$ method was used to quantify transcript levels (Livak and Schmittgen 2001). The data were obtained from 3 independent biological replicates. The mean transcript values were Log2-normalized to generate heatmap using TB Tools (Chen et al. 2020).

Agrobacterium-mediated infiltration in *N. benthamiana* leaves

Agrobacterium cells EHA109 harboring each relevant construct were grown in LB medium with the appropriate antibiotics until they reached an absorbance at 600 nm of 0.6. Bacterial cells were pelleted, washed, and resuspended in infiltration medium (10 mM 2-(N-morpholino) ethane sulfonic acid, 10 mM $MgCl_2$, and 0.15 mM acetosyringone, pH 5.6). The resulting suspension was incubated at 25 °C for 4 to 6 h, and then infiltrated into the leaves of 1-month-old *N. benthamiana* plants. Infiltrated plants were kept overnight at 24 °C in the dark, followed by one 16-h light/8-h dark cycle. After 48 h, fluorescence in the leaf samples was observed using a laser scanning confocal microscope (Leica TCS-SP8) equipped with argon laser with excitation sources using a 20× objective.

Subcellular localization of SKIP31

The coding sequence of *SKIP31* was cloned in-frame and downstream of the sequence encoding YFP (yielding *YFP-SKIP31*) in the pSITE-3CA vector. The resulting plasmid was transformed to Agrobacterium strain EHA109 and infiltrated in the lower epidermis of *N. benthamiana* leaves as above. NLS-RFP and AtCESA-RFP were used as markers for the nucleus and the plasma membrane, respectively. Fluorescence was observed after 48 h by laser scanning confocal microscopy (Leica TCS-SP8) equipped with argon laser with excitation sources using a 20× objective.

Yeast 2-hybrid

To screen the Y2H library for the identification of SKIP31 substrates, the *SKIP31* coding sequence was amplified and cloned into the bait vector pGBKT7-BD); the resulting plasmid was transformed into the Y2H Gold strain (Clonetech) using an EZ-Yeast Transformation Kit (MP Biomedicals) according to the manufacturer's instructions. The transformed pGBKT7-BD): SKIP31 cells were then mated to a normalized Arabidopsis cDNA library transformed yeast Y187 cells and screened according to the technique outlined by Rao et al. (2018).

For SKIP31-ASKs pairwise interactions, the coding sequences of the indicated *ASKs* (*ASK1* to *ASK15* and *ASK17 to ASK19*) were individually amplified and cloned in a Gateway compatible destination vector pDEST-GADT7 (AD) by LR recombination. Similarly, for the SKIP31-JAZ interactions, the coding sequences of each of the indicated 12 *JAZs* (*JAZ1 to JAZ12*) were individually cloned in the pDEST-GADT7vector. To identify domain for the interaction, full length and truncated SKIP31 was cloned in the pDEST-GBKT7.To assess protein interaction, the generated pDEST-GADT7 (AD) and pDEST-GBKT7 (BD)—constructs

were co-transformed into Y2H gold strain as described by Rao et al. (2018). The primers used in the study are listed in Supplemental Data Set 1.

Bimolecular fluorescence complementation

The full-length coding sequence of *SKIP31* and that of the indicated *JAZ* (*JAZ1*, *JAZ6*, *JAZ10*, and *JAZ11*) were cloned in-frame of the sequence encoding the N-terminal half of YFP or the C-terminal half of YFP, respectively, in the CD3-1648 and CD3-1651 vectors. Each construct was then transformed into Agrobacterium EHA109 strain and then *SKIP31-nYFP* was co-infiltrated together with *JAZ1-cYFP* or *JAZ6-cYFP* or *JAZ10-cYFP* or *JAZ11-cYFP* into the leaves of 1-month-old *N. benthamiana* plants as described previously, along with appropriate controls. Similarly, the full-length coding sequences of *JAZ6* and *JAZ11*, *ABI3* and *ABI5* were cloned in-frame with the sequence encoding the C-terminal half of YFP or the N-terminal half of YFP, respectively, in the CD3-1651 and CD3-1648 vectors. Each construct was then transformed into Agrobacterium EHA109 strain and *ABI3-nYFP* or *ABI5-nYFP* was co-infiltrated with *JAZ6-cYFP* or *JAZ11-cYFP* into the leaves of 1-month-old *N. benthamiana* plants. Fluorescence was observed after 48 h under a laser scanning confocal microscope (Leica TCS-SP8) equipped with argon laser with excitation sources using a 20× objective. The primers used in the study are listed in Supplemental Data Set 1.

Bacterial production and purification of recombinant proteins

The *SKIP31* cDNA was subcloned into the EcoRI and PstI sites of the bacterial expression vector pMAL-c-2X to produce MBP-tagged SKIP31, while JAZ cDNAs were subcloned into various pET vectors (pET23b: JAZ1 [NdeI/XhoI], pET23d: JAZ6 [NcoI/XhoI] and pET23b: JAZ11[NdeI/HindIII]) to obtain His-tagged JAZ recombinant proteins. The resulting plasmids were introduced into the host strain *Escherichia coli* BL21-DE3. Transformed *E. coli* cells were grown in LB medium at 37 °C until they reached an absorbance at 600 nm of 0.5, followed by induction with 0.5 mM IPTG for 8 h at 37 °C. The bacterial cells were collected by centrifugation (8,000×g for 10 mins at 4 °C) and lysed by sonication in a buffer containing 20 mM Tris-HCl pH 7.6, 150 mM NaCl, and 10 mM β-mercaptoethanol, containing 1× bacterial protease inhibitor cocktail (Sigma). The extracts were analyzed by 12% SDS-PAGE (Supplemental Fig. S10) and purified using an affinity column chromatography. For purification of MBP-tagged SKIP31, dextrin amylose MBP resin (G Biosciences) was used; for His-tagged JAZ proteins, Ni-NTA beads (Qiagen) were used. The primers used in the study are listed in Supplemental Data Set 1.

Immunoblot analysis

Immunoblot analysis was carried out using suitable antibodies as described by Verma et al. (2013). Protein samples were separated on 12% SDS-PAGE before being electroblotted onto a PVDF membrane. The membranes were probed with suitable and/or indicated antibodies and developed using Clarity Western ECL substrate (BioRad). The antibodies used in the study are as follows: Anti-MBP, 1:10,000 (Agrisera, Cat No.#AS153039), Anti-HIS, 1:5,000 (Sigma, Cat No.#SAB1306082-400ul) Anti-ubiquitin, 1:1,000 (Enzo life sciences Auto-ubiquitylation kit, Cat No.#BML-UW0970), Anti-Rabbit (secondary antibody), 1:5,000 (Amherst Cat No.#NA934-1 mL), and Anti-mouse (secondary antibody), 1:5,000 (Pierce Ab Thermo, Cat No.#31430).

Pull-down assay

For in vitro pull-down assays, recombinant MBP and MBP-SKIP31 were immobilized as baits on dextrin amylose resin before being incubated with recombinant His-tagged JAZ proteins (JAZ6-His and JAZ11-His). After the prescribed washing steps, the complexes were selectively eluted using 10 mM maltose. The eluted complex was divided into 2 parts, resolved by SDS-PAGE, and analyzed by immunoblotting. Two sets of immunoblot assays were performed using anti-MBP (1:10,000 dilution; Agrisera CatNo.# AS153039) and anti-HIS (1:5,000 dilution; Sigma CatNo.# SAB1306082-400 μL) antibodies, respectively.

In vitro ubiquitination assay

For in vitro ubiquitination assays, a ubiquitinylating kit was used (Enzo life sciences #BML-UW0970). All desired components were mixed into the reaction according to the manufacturer's protocol, and incubated at 30 °C for 1.5 h. The reactions were terminated by adding 5xSDS loading dye, followed by immunoblotting analysis using the indicated antibodies (Anti-HIS, 1:5,000 [Sigma, Cat No.#SAB1306082-400ul]; Anti-ubiquitin, 1:1,000 [Enzo life sciences, Cat No.#BML-UW0970]).

Cell-free degradation assay

For cell-free degradation assay, 14-d-old seedlings from WT, *coi1*, *SKIP31*-RNAi grown on half-strength MS plates were used as the source of 26S proteasome machinery. Extracts were prepared in extraction buffer (25 mM Tris-HCl pH 7.5, 10 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, and 10 mM ATP). Five micrograms of recombinant His-tagged JAZ (JAZ6-His or JAZ11-His) was incubated with a cell-free crude extract (50 to 100 μg crude protein) alone or with 5 μg MBP-SKIP31 (as a source of E3 ligase) for the indicated time points in the presence or absence of 1× proteasome inhibitor MG132 (Sigma, CatNo.#C2211). The reactions were terminated by adding 5xSDS sample buffer followed by immunoblotting analysis. The membranes were probed with an anti-His antibody (1:5,000 dilution, Sigma CatNo.# SAB1306082-400ul) to detect the levels of His-tagged JAZ proteins. Another blot with the same reaction setup was probed with an anti-MBP antibody (1:10,000 dilution; Agrisera Cat No.#AS153039) to detect the levels of MBP-SKIP31. Equal loading of the sample was determined by Ponceau staining of Rubisco large subunit.

Seed germination assay

The protocol for germination assay for all transgenic, mutants, and WT seeds followed the method described by Rao et al. (2018) and Majee et al. (2018) with minor modifications. All seeds used in these experiments were harvested on the same day from dry mature siliques. The seeds were surface sterilized with 0.3% (w/v) sodium hypochlorite and plated on aqueous medium (0.6% [w/v] agar, 10 mM MES pH 5.7) or half-strength MS medium. After stratification for 3 d, seed germination was initiated in growth chambers (22 ± 2 °C with a 16-h light/8-h dark photoperiod and a light intensity of 120 μmol/m^2/s); the germination rate was scored on the 7th day. Seed germination was considered when radicle protrusion was observed. For checking the germination of developing seeds (green seeds, 12 to 15 DAF), seeds were carefully separated from green siliques on wet Whatman filter paper. For seed dormancy experiments, freshly harvested seeds were tested for germination without stratification.

For the seed germination experiments, mean and significance were calculated from at least 3 biological replicates, harvested from plants grown at 3 different times. For each biological replicate, all genotypes were grown in identical conditions and harvested at the same time, and seeds were pooled from at least 20 independent plants; each seed batch was tested at least 3 times as technical replicates or as indicated. The value of each biological replicate was calculated as the average of over 3 technical replicates by analysis of 150 seeds (3 × 50 in each of the technical replicates) or as indicated.

Desiccation tolerance

Dry mature seeds were kept at 25 °C in a dry seed storage cabinet (15% RH) for the required times (0 to 8 wk), which allowed seeds to undergo natural desiccation. Developing seeds (S5, 13 to 15 DAF) were dried at 25 °C for 2 d in the seed storage cabinet. Seeds were then hydrated with sterile water and kept at 4 °C for 3 d for stratification before proceeding for germination.

Tetrazolium staining and formazan estimation

For seed viability assays, tetrazolium staining was carried out as described in previous studies (Verma and Majee 2013; Hazra et al. 2022). Surface-sterilized seeds were treated with 1% (w/v) tetrazolium and were incubated at 30 °C for 48 h. Photographs were taken under a Nikon stereo zoom microscope.

Formazan formation in the tetrazolium assay was quantified using a spectrophotometric assay using 3 biological replicates for each genotype as reported by Bafoil et al. (2018) with minor modifications. Seeds were incubated for 24 h in tetrazolium red at 28 °C and formazan production was assayed by measuring the absorbance at 492 nm of 100 crushed seeds in 95% (v/v) ethanol using ethanol as blank. Formazan absorbance, upon tetrazolium staining, was used to measure the seed vigor and/or viability.

Controlled deterioration treatment

The CDT was carried out according to Salvi et al. (2016). The moisture content of the seeds was raised to 22 ± 2% before they were exposed to a higher temperature. Seeds were tested for viability and germination rates after being incubated at 45 °C for 4 d at 70% RH. Experiments were conducted in triplicate, with each batch containing 50 seeds.

Phytohormone quantification and GC-MS analysis in Arabidopsis seeds

Phytohormones were quantified from mature Arabidopsis seeds following a method described by Vadassery et al. (2012) with minor modifications. Fifty milligrams seeds were ground in liquid nitrogen and then homogenized and extracted in methanol containing internal standards (OIChemIm Ltd, http://www.olchemim.cz/). The extracted supernatant (12,000×g for 15 mins at 4 °C) was lyophilized, reconstituted in 500 μL methanol, and finally injected into an AB Sciex QTRAP 6500 Exion AD LC system (AB Sciex) with an Acquity UPLC BEH C18 column (2.1 100 mm, 1.7 μm, Waters) and turbo spray ion drive electrospray ionization (ESI) source.

For metabolite extraction from Arabidopsis seeds, the method by Kundu et al. (2018) was followed with minor modifications. Seeds were ground in liquid nitrogen and homogenized in 1 mL chilled methanol (HPLC Grade), and then adonitol (20 mg/mL) was added as an internal standard to the extraction. The samples were mixed, vortexed, and incubated at 70 °C for 15 min. Subsequently, 800 μL of HPLC grade water and 600 μL of chloroform were added. After centrifugation at 10,000 g for 15 min at 25 °C, the supernatant was collected and lyophilized until the solvent was completely evaporated. For derivatization, dried lyophilized samples were resuspended in 80 μL of methoxyamine hydrochloride in 20 mg/mL pyridine and then incubated at 30 °C for 90 min. Trimethylsilylation was accomplished using 80 μL of MSTFA [N-methyl-N-(trimethylsilyl) trifluoroacetamide] followed by incubation at 37 °C for 30 min. Samples were then centrifuged at 14,000 g for 5 min at 25 °C, and supernatants were used for analysis. GC-MS analysis was performed using Shimadzu QP2010 Ultra using the Rtx-5Sil-MS column (0.25 mm × 30 m × 0.25 μm, Restek Corporation, USA). The temperature program consisted of 60 °C isothermal heating for 2 min, followed by a ramp rate of 5 °C/min to 250 °C, withhold time 5 min, and subsequently a final ramp rate of 10 °C/min to 280 °C, withhold time 15 min. The chromatograms were examined using GC-MS solution software. The NIST12 library was used for peak identification.

GUS staining

Tissues were incubated overnight in a GUS staining solution (100 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 50 mM $K_3Fe(CN)_6$, 50 mM $K_4Fe(CN)_6$ and 1 mM X-gluc [Gold biotechnology, St. Louis, MO]) at 37 °C.

To remove chlorophyll, the samples were incubated in GUS destaining solution (ethanol: glacial acetic acid: water, 3:1:1, v/v/v) and photographed under a Nikon stereo zoom microscope.

Scanning electron microscopy

The mature seeds of the indicated genotypes were harvested from the plants and dried under desiccator for 24 h. They were mounted on separate aluminum stubs randomly and set under the SEM. Images were recorded on a ZEISS EVO LS 10 SEM with electron high distance at 20.00 kV, working distance 8.0 mm at 250 × and 1,000 × magnification.

Microtome sectioning

Microtome sections were produced out according to the protocol described by Kladnik (2013) with minor modifications. Arabidopsis seeds were fixed overnight in a fixative solution (10% formaldehyde, 5% glacial acetic acid, 50% ethanol, and 35% water) and then dehydrated in a graded series of tetrabutanol (TBA) with ethanol, and finally with 100% TBA. The dehydrated tissue was embedded in paraffin wax (paraplast plus) for 2 d. Paraffin wax was changed every 12 h during this period, followed by manual casting of the wax blocks. Using a rotary microtome (Leica RM2255; Leica), the wax blocks were sectioned at 5-µm thickness. The slices were stored at 4 °C until used for dewaxing. After dewaxing in a series of xylene and water, microscopy images were taken under a Nikon stereo-zoom microscope.

Dual-luciferase reporter assay

The promoter region of *SKIP31* was cloned upstream of *LUC* in the pGreenII 0800-LUC vector to generate the *proSKIP31:LUC* reporter, with *Renilla* luciferase (*REN*) as an internal control (Hellens et al. 2005). Subsequently, the coding sequences of *JAZ* (*JAZ6* and *JAZ11*) and *ABI5* were individually cloned in-frame with the *YFP* sequence into the pEG101 vector for effector constructs. Combinations of the indicated plasmids were introduced into *N. benthamiana* leaves via Agrobacterium-mediated infiltration. Relative LUC activity was determined using a Dual-luciferase reporter assay kit (Promega) according to the manufacturer's instructions by measuring the activities of firefly LUC and REN with a spectrofluorometer (Polar Star omega microplate reader, BMG Labtech, model no. BMG-CO2R). The LUC/REN ratios were calculated. The primers used in the study are listed in Supplemental Data Set 1.

Carbohydrate, protein, and lipid analysis in Arabidopsis seeds

The contents of mature seeds for carbohydrates, protein, and lipids were analyzed as 3 biological replicates grown and harvested at 3 different times; results are expressed on a per seed weight basis. For each replicate, same-aged seed population was harvested from a pool of 20 plants. A protocol from Leach and Braun (2016) was followed for sucrose and starch extraction from Arabidopsis seeds with some minor modifications. Briefly, 40 mg ± 5 mg of Arabidopsis seeds were weighed and ground in liquid nitrogen. To the powder, 0.5 mL MCW (methanol:chloroform:water, 12:5:3, v/v/v) was added, vortexed, and incubated in a 50 °C water bath for 30 min. The samples were centrifuged for 5 min at 10,000 g at room temperature and the supernatant was transferred into a 1.5-mL microcentrifuge tube (MCT) and kept on ice. Further, 0.5 mL of MCW extraction buffer was again added to the pellet, and the extraction procedures were repeated 3 more times and the obtained supernatants were pooled. The pooled supernatant extracts were used for sucrose estimation with a Megazyme assay kit (Megazyme Sucrose Assay Kit [K-SUFRG] as per manufacturer's protocol).

The residue tissue pellet after the third extraction was used for total starch analysis. The solubilized starch solution was diluted by adding 50 µL of the slurry to 950 µL of 100 mM sodium acetate, pH 5.0. The diluted solution was incubated with 100 µL of α-amylase (supplied with the Megazyme Total Starch Assay Kit; K-TSTA-100A) working solution in a boiling water bath for 15 min. Further, 20 µL of amyloglucosidase (supplied with the Megazyme Total Starch Assay Kit; K-TSTA-100A) was added, vortexed, and incubated for 1 h at 50 °C. The reactions were then centrifuged at 10,000 g for 5 min at room temperature. The supernatant was then transferred to a new tube and used for starch estimation. The starch estimation was conducted as per the manufacturer's protocol for the Megazyme Total Starch Assay Kit (K-TSTA-100A).

For total SSP extraction, 20 ± 5 mg of Arabidopsis seeds were homogenized in 0.5 mL of protein extraction buffer (50 mM HEPES pH 7.5, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 10% [v/v] ethylene glycol in the presence of Polyclar) (Baud et al. 2002; Chen et al. 2012). After 5 min in boiling water, the homogenates were centrifuged at 15,000 g for 10 min at 4 °C. The obtained supernatant was used for estimating the content of SSPs. Three biological replicates were used to quantify protein content in the fractions using the Bradford reagent assay (Bradford 1976).

The procedure described by Bligh and Dyer (1959) was used to extract lipids from seeds (Siloto et al. 2006). Briefly, 40 ± 5 mg Arabidopsis seeds were homogenized in liquid nitrogen and then treated with 5 mL of isopropanol at 70 °C for 10 min. Lipids were extracted with 3 extractions of chloroform, methanol, and water biphasic solutions after the isopropanol was evaporated under nitrogen flow (methanol: $CHCl_3$: H_2O). A mix of methanol: $CHCl_3$: H_2O (2:2:1.8, v/v/v) was used for the first extraction, and a mix of methanol: $CHCl_3$: H_2O (1:2:0.8, v/v/v) was used for the second and third extractions. Under nitrogen flow, the lipid fractions were collected and the solvents were evaporated. After drying the samples in a desiccator for 24 h, total lipids were measured using the gravimetric method (determine the quantity of extracted lipids based on its mass after evaporating all its solvent) as described by Bligh and Dyer (1959).

Statistical analysis

All data in this study are shown as means with SE. SE was calculated from 3 replicates, as mentioned in the legends. Statistical analysis was conducted by 1-way ANOVA and student's *t*-test. Duncan's Multiple Range Test ($\alpha = 0.01$ or as indicated) was performed using the SPSS program (SPSS, Chicago, IL, USA) to test for statistical significance. The Student's *t*-tests were calculated from GraphPad by DotMatics. Statistical data are provided in Supplemental Data Set 3.

Accession numbers

Sequence data from this article can be found in the GenBank data libraries under the following accession numbers: *SKIP31*, At5g45360; *JAZ1*, At1g19180; *JAZ2*, At1g74950; *JAZ3*, At3g17860; *JAZ4*, At1g48500; *JAZ5*, At1g17380; *JAZ6*, At1g72450; *JAZ7*, At2g34600; *JAZ8*, At1g30135; *JAZ9*, At1g70700, *JAZ10*, At5g13220; *JAZ11*, At3g43440; *JAZ12*, At5g20900; *ASK1*, At1g75950; *ASK2*, At5g42190; *ASK3*, At2g25700; *ASK4*, At1g20140; *ASK5*, At3g60020; *ASK6*, At3g53060; *ASK7*, At3g21840; *ASK8*, At3g21830; *ASK9*, At3g21850; *ASK10*, At3g21860; *ASK11*, At4g34210; *ASK12*, At4g34470; *ASK13*, At3g60010; *ASK14*, At2g03170; *ASK15*, At3g25650; *ASK17*, At2g20160; *ASK18*, At1g10230; *ASK19*, At2g03160; *RD29A*, At5g52310; *RD29B*, At5g52300; *RAB18*, At5g66400; *DREB2A*, At5g05410; *NCED3*, At3g14440; *NCED6*, At3g24220; *EM1*, At3g51810; *EM6*, At2g40170; *ABI3*, At3g24650; *ABI4*, At2g40220; *ABI5*, At2g36270; *COI1*, At2g39940; *GolS1*, At2g47180; *SUS3* At4g02280, *CRU3*, At4g28520; *2S2*, At4g27150.

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Author contributions

M.M. and V.V. designed and conceptualized the idea of the article. V.V. has done all the major experiments and analyzed the data. A.H. assisted in real-time, silique cutting and microscopy. V.R. has done the Y2H library screening. S.G., N.U.K, R.K.A, and S.Ga. helped in *Nicotiana* plant growth care and seed harvesting. V.V. and M.M. wrote the paper and created the figures. M.M. supervised and funded the research. All authors read and approved the final manuscript. M.M. agrees to serve as the author responsible for contact and ensures communication.

Supplemental data:

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

Supplemental Figure S1. *SKIP31* genomic organization, protein domains, and structure.

Supplemental Figure S2. Histochemical GUS staining in different organs of *proSKIP31:GUS* transgenic lines and *SKIP31* transcript accumulation during seed development and germination.

Supplemental Figure S3. Transcript analysis and seed germination percentage in *skip31* mutants.

Supplemental Figure S4. Generation, screening, and confirmation of *SKIP31*-OE and *SKIP31*-RNAi transgenic lines.

Supplemental Figure S5. Seed germination rates in generated *SKIP31* transgenic lines.

Supplemental Figure S6. Seed desiccation tolerance at the S5 developmental stage of seeds from WT, the *skip31* mutant and *SKIP31*-RNAi transgenic lines.

Supplemental Figure S7. *skip31* mutants and *SKIP31*-RNAi lines show defects in seed maturation and germination.

Supplemental Figure S8. Ectopic overexpression of *SKIP31* successfully rescues the phenotype of the *skip31* mutant CS848440.

Supplemental Figure S9. *SKIP31* overexpression improves seed vigor after CDT. **Supplemental Fig. S10.** Germination rates of the indicated genotypes in response to GA treatment.

Supplemental Figure S11. Bacterial production of MBP-SKIP31 and JAZ-His in *Escherichia coli* BL21-DE3.

Supplemental Figure S12. In vitro ubiquitination assay of JAZ proteins via SKIP31.

Supplemental Figure S13: Cell-free degradation assay showing the 26S proteasomal degradation of JAZ proteins.

Supplemental Figure S14. Transcript analysis and seed germination rates in *JAZ* full-length OE lines.

Supplemental Figure S15. Transcript level analysis in *JAZ6Δjas and JAZ11Δjas* overexpression lines.

Supplemental Figure S16. The *JAZΔjas* OE lines mimic *SKIP31* RNAi lines.

Supplemental Figure S17. *JAZΔjas* OE lines show defects in later stages of seed maturation.

Supplemental Figure S18. *coi1-1* shows no defects in seed maturation or viability.

Supplemental Figure S19. BIFC interaction studies between JAZ proteins and ABI3 and ABI5.

Supplemental Figure S20. Heatmap showing the differential pattern of transcript accumulation of several chosen genes important for seed maturation and ABA response in the indicated genotypes.

Supplemental Figure S21. Transcript levels of *CRU3* and *2S2* in the indicated genotypes at the S5 seed developmental stage.

Supplemental Figure S22. In silico analysis of the *SKIP31* promoter.

Supplemental Figure S23. Heatmap showing the differential pattern of transcripts of several genes important for seed maturation and ABA response in the WT Ws-2 and the *abi5-1* mutant.

Supplemental Figure S24. Ectopic overexpression of *ABI5* partially rescues the phenotype of *SKIP31*-RNAi lines.

Supplemental Figure S25. Seed dormancy phenotypes of freshly harvested seeds of Arabidopsis indicated WT, mutants and transgenic lines.

Supplemental Data Set 1. Primers used in this study.

Supplemental Data Set 2. Normalized RT-qPCR values used to generate the heatmaps.

Supplemental Data Set 3. Summary of statistical analyses.

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Conflict of interest statement. None declared.

Data availability

All data are available in the main text and in the Supplemental data.

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