The RING E3 Ligase KEEP ON GOING Modulates JASMONATE ZIM-DOMAIN12 Stability[1[OPEN]]

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Jasmonate (JA) signaling in plants is mediated by the JASMONATE ZIM-DOMAIN (JAZ) proteins that repress the activity of several transcription factors regulating JA-inducible gene expression. The hormone JA-isoleucine triggers the interaction of JAZ repressor proteins with the F-box protein CORONATINE INSENSITIVE1 (COI1), part of an S-phase kinase-associated protein1/Cullin1/F-box protein COI1 (SCF^{COI1}) E3 ubiquitin ligase complex, and their degradation by the 26S proteasome. In Arabidopsis (*Arabidopsis thaliana*), the JAZ family consists of 13 members. The level of redundancy or specificity among these members is currently not well understood. Here, we characterized JAZ12, encoded by a highly expressed *JAZ* gene. JAZ12 interacted with the transcription factors MYC2, MYC3, and MYC4 in vivo and repressed MYC2 activity. Using tandem affinity purification, we found JAZ12 to interact with SCF^{COI1} components, matching with observed in vivo ubiquitination and with rapid degradation after treatment with JA. In contrast to the other JAZ proteins, JAZ12 also interacted directly with the E3 RING ligase KEEP ON GOING (KEG), a known repressor of the ABSCISIC ACID INSENSITIVE5 transcription factor in abscisic acid signaling. To study the functional role of this interaction, we circumvented the lethality of *keg* loss-of-function mutants by silencing *KEG* using an artificial microRNA approach. Abscisic acid treatment promoted JAZ12 degradation, and *KEG* knockdown led to a decrease in JAZ12 protein levels. Correspondingly, KEG overexpression was capable of partially inhibiting COI1-mediated JAZ12 degradation. Our results provide additional evidence for KEG as an important factor in plant hormone signaling and a positive regulator of JAZ12 stability.

The JASMONATE ZIM DOMAIN (JAZ) proteins are central in the signal transduction cascade triggered by the plant hormone (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile). In the presence of JA-Ile, they form a coreceptor complex with the F-box protein CORONATINE INSENSITIVE1 (COI1; Fonseca et al., 2009, Sheard et al., 2010). COI1 forms part of the E3 ubiquitin ligase complex S-phase kinase-associated protein1 (Skp1)/Cullin1/F-box protein COI1 (SCF^{COI1}) that mediates the ubiquitination of the interacting JAZ, which then leads to degradation of the JAZ protein by the proteasome (Chini et al., 2007; Thines et al., 2007). Within seconds after the perception of JA-Ile, JAZ proteins begin to degrade (Pauwels et al., 2010).

JAZ proteins function as repressors of specific transcription factors in the absence of JA-Ile (Niu et al., 2011; Pauwels and Goossens, 2011; Zhu et al., 2011; Hu et al., 2013; Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013; Fonseca et al., 2014). The perception of jasmonates (JAs) thus leads to the derepression of these transcription factors, followed by a rapid reprogramming of gene expression (Pauwels et al., 2008; Attaran et al., 2014). One of the best characterized transcription factors repressed by JAZ proteins is the basic helix-loop-helix protein MYC2 (Lorenzo et al., 2004; Chini et al., 2007; Kazan and Manners, 2012). MYC2-JAZ interactions are predominantly mediated by the C-terminal Jas domain on JAZ proteins, which is also the site of JA-Ile and COI1 interaction (Katsir et al., 2008; Melotto et al., 2008; Sheard et al., 2010). The molecular mechanism by which JAZ proteins repress transcription factor activity includes recruitment of the corepressor TOPLESS (TPL), either directly or through the adaptor protein NOVEL INTERACTOR OF JASMONATE ZIM DOMAIN (NINJA; Pauwels et al., 2010; Acosta et al., 2013).

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There are 13 JAZ proteins in Arabidopsis (*Arabidopsis thaliana*; Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Thireault et al., 2015). It is assumed that there is a high level of redundancy, as indicated by the fact that many single *jaz* loss-of-function mutants do not have a phenotype (Thines et al., 2007). However, some distinct properties of JAZ proteins are being discovered. Whereas most of the JAZ proteins interact with NINJA for interaction with the corepressor TPL, JAZ7 and JAZ8 do not (Pauwels et al., 2010). JAZ7 and JAZ8 have an ethylene-responsive element binding factor-associated amphiphilic repression motif themselves, which mediates direct interaction with TPL (Shyu et al., 2012). Moreover, JAZ8 lacks a canonical degron; therefore, it is unable to associate strongly with COI1 in the presence of JA-Ile and, thus, is resistant to JA-mediated degradation (Shyu et al., 2012). Other examples are JAZ1 and JAZ10, which contain, besides the C-terminal Jas domain, an additional Jas-like domain at their N terminus called the cryptic MYC2-interacting domain, which also mediates interaction with MYC2 (Moreno et al., 2013; Goossens et al., 2015). Possibly, this domain explains the dominant JA-insensitive phenotype when overexpressing *JAZ10.3* and *JAZ10.4* splice variants. These variants lack part of the Jas domain and lose COI1 interaction, but they can still interact with MYC2 (Moreno et al., 2013).

Several studies have localized GFP-fused JAZ proteins to the nucleus (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Chung and Howe, 2009; Grunewald et al., 2009; Shyu et al., 2012; Withers et al., 2012). These observations are consistent with JAZ proteins functioning to repress transcription factors by promoting binding to the corepressor TPL. Recently, it was proposed that the nuclear localization of JAZ proteins is dependent on their interaction with MYC2. In a *myc2* loss-of-function mutant, yellow fluorescent protein (YFP)-JAZ9 is partially mislocalized to the cytoplasm (Withers et al., 2012). These results suggest that JAZ function is at least partially regulated by subcellular localization.

The pathway of JA signaling resembles in many ways that of ABSCISIC ACID-INSENSITIVE5 (ABI5) and related ABSCISIC ACID-RESPONSIVE ELEMENT BINDING FACTOR (ABF) basic leucine zipper (bZIP)-type transcription factors. G-box elements in promoters of JA- and abscisic acid (ABA)-responsive genes are recognized by basic helix-loop-helix- and bZIP-type transcription factors, respectively. These transcription factors then interact indirectly or directly with NINJA or the NINJA-related ABI5-binding proteins (Garcia et al., 2008). The latter also function as TPL adaptor proteins (Pauwels et al., 2010). Furthermore, several points of cross talk between JA and ABA signaling have been reported, possibly underlying the complex interplay between these pathways in disease resistance (Anderson et al., 2004). For instance, the genes encoding the ABA receptors PYRABACTIN RESISTANCE-LIKE4 (PYL4) and PYL5 are up-regulated by JA, and their mutants show altered JA responses (Lackman et al., 2011). Conversely, MYC2 also plays an important role in this cross talk, as it had been identified as a positive regulator of ABA signaling (Abe et al., 2003; Lorenzo et al., 2004). Correspondingly, overexpression of a mutant *MYC2* version that cannot be fully repressed by JAZ proteins leads to ABA hypersensitivity (Goossens et al., 2015).

The protein KEEP ON GOING (KEG) is an additional regulator of the bZIP-type transcription factors ABI5, ABF1, and ABF3 (Stone et al., 2006; Chen et al., 2013). This RING-type ubiquitin E3 ligase interacts directly with ABI5 and is a negative regulator of ABA signaling (Liu and Stone, 2010, 2013). In the absence of ABA, KEG ubiquitinates ABI5, leading to its proteasomal degradation (Stone et al., 2006; Liu and Stone, 2010). ABA treatment, however, promotes KEG self-ubiquitination and degradation, leading to an increase in ABI5 levels (Liu and Stone, 2010). Intriguingly, in contrast to the nuclear ABI5 subcellular localization, KEG is present in the trans-Golgi network (TGN; Gu and Innes, 2011) and is proposed to be a regulator of post-Golgi trafficking, including the secretion of apoplastic defense proteins (Gu and Innes, 2012). However, when inactivating the RING domain of KEG or mutating a conserved Lys in ABI5, an interaction between KEG and ABI5 can be observed outside the nucleus in the cytoplasm and at the TGN (Liu and Stone, 2013). This leads to a model in which cytoplasmic KEG activity regulates nuclear ABI5 levels because the latter is transported between these compartments (Liu and Stone, 2013).

Here, we characterized JAZ12 as a representative JAZ protein that interacts with SCF^{COI1} in a JA-Ile-dependent manner and negatively regulates MYC2. JAZ12 was

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L.P., A.R., J.Go., A.N.D., H.L., Y.Gu., J.Ge., and M.B. performed the experiments; R.D.C. and R.V.B. provided technical assistance; J.V.L. analyzed the data; K.G., G.D.J., R.S., S.S., R.W.I., and J.C. supervised the experiments; L.P. and A.G. conceived the project and wrote the article with contributions of all the authors.

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found to be ubiquitinated in vivo and rapidly degraded upon JA treatment. However, in contrast to other JAZ proteins, JAZ12 interacted through its Jas domain directly with the HERC2-like (for HECT and RCC1-like) repeat domain of KEG. Intriguingly, KEG acted as a positive regulator of JAZ12 protein levels. ABA treatment increased JAZ12 degradation, and knocking down *KEG* led to lower JAZ12 levels, whereas overexpression of *KEG* partially prevented the COI1-mediated degradation of JAZ12.

RESULTS

Isolation of COI1 and JAZ12 Protein Complexes

Previously, we reported that tandem affinity purification (TAP) of the JAZ protein JAZ1 in Arabidopsis cell cultures copurified the F-box protein COI1 when cells were treated for 1 min with JA (Pauwels et al., 2010). Here, we generated Arabidopsis cells expressing *COI1* tagged on its C terminus with the protein G/streptavidin-binding peptide (GS) tag. After mock treatment with ethanol, we could purify the SCF components ARABIDOPSIS SKP-LIKE1 (ASK1), ASK2, and CULLIN1 (CUL1; Table I; Supplemental Data Set S1). When treated with 50 μM JA for 1 min, JAZ12 and NINJA were found. No peptides of other JAZ proteins were detected. RNA sequencing (RNA-Seq) analysis revealed that *JAZ12* was an abundantly expressed *JAZ* gene both in this system and in Arabidopsis seedlings (Fig. 1A), likely explaining why it was recovered preferentially over other JAZ proteins.

Next, we obtained *JAZ12-GS*-expressing cells and affinity-purified JAZ12-associated proteins from cells. In mock-treated cells, JAZ12 copurified with the JAZ proteins JAZ10 and JAZ2, NINJA, and the transcription factors MYC2, MYC3, and MYC4 (Table I). When treated briefly with JA, we detected COI1, ASK1, and ASK2 peptides (Table I), indicating that the SCF^{COI1} complex associated with JAZ12.

Using our TAP approach for Arabidopsis seedlings (Van Leene et al., 2015), we could show that JAZ12 formed a similar complex as in the JA-treated suspension cells, thus associating with SCF^{COI1} even in the absence of exogenously added JA. Only one difference was noted: JAZ6 but not JAZ10 copurified. In the TAP performed with seedlings, a JAZ12 peptide modified with di-Gly could be found, pinpointing that Lys-169 is a putative in vivo modification site for ubiquitin or a ubiquitin-like protein site (Supplemental Data Set S1; Kirkpatrick et al., 2005). Finally, in all JAZ12 TAP experiments, a previously unassociated protein was detected, the E3 ubiquitin ligase KEG, which has previously been linked to ABA signaling (Stone et al., 2006).

Table I. *Overview of the proteins purified by TAP with JAZ12 and COI1*

Proteins were identified using peptide-based homology analysis of mass spectrometry data. Background proteins were withdrawn based on the frequency of occurrence of copurified proteins in a large GS TAP data set (Van Leene et al., 2015). Numbers indicate the times the prey was identified in two experiments per column (−/+JA) with COI1 and four experiments per column (−/+JA) with JAZ12 for cells. Only one experiment was performed for seedlings. Only preys identified in at least two experiments were retained. AGI, Arabidopsis Genome Initiative identifier. –, Prey was not identified in this experiment.

AGI	Protein	JAZ12			COI1	
		Cells	Cells	Seedlings	Cells	Cells
		−JA	+JA	−JA	−JA	+JA
AT5G20900	JAZ12	4	4	1	–	2
AT5G13220	JAZ10	2	2	–	–	–
AT1G72450	JAZ6	–	–	1	–	–
AT1G74950	JAZ2	4	3	1	–	–
AT4G28910	NINJA	4	4	1	–	2
AT4G17880	MYC4	4	4	1	–	–
AT1G32640	MYC3	4	4	1	–	–
AT1G32640	MYC2	4	4	1	–	–
AT2G39940	COI1	–	4	1	2	2
AT1G75950	ASK1	–	4	1	2	2
AT5G42190	ASK2	–	3	1	2	2
AT4G02570	CUL1	–	2	1	2	2
AT5G12140	CYS1	2	2	–	–	–
AT5G13530	KEG	4	4	1	–	–

JAZ12 Is a Typical JAZ Protein

The protein sequence of JAZ12 is very representative of the JAZ protein family. Besides the ZIM and Jas domains, no other conserved protein sequences have been reported for JAZ12 (Cuéllar Pérez et al., 2014). The best-known protein-protein interactions of the JAZ proteins could be confirmed for JAZ12 by TAP (Table I). To verify this further, we first tested direct interaction with COI1 using the LexA-based yeast two-hybrid (Y2H) system, which has been used previously to show interactions between COI1 and JAZ9 (Melotto et al., 2008). Both JAZ9 and JAZ12 interacted with COI1 in the presence of 50 μM coronatine (COR), a JA-Ile mimic (Fig. 1B). Next, we tested the JA-mediated degradation of JAZ12. Cells expressing GS-tagged *JAZ12* were treated with 50 μM JA or ethanol for 30 min, 1 h, and 3 h. Already at the first time point, JAZ12-GS protein was nearly undetectable (Fig. 1C).

Y2H analyses also confirmed the direct interaction of JAZ12 with MYC2 (Fig. 1D). We have shown previously that a reporter construct with the JA-inducible *LIPOXYGENASE3* (*LOX3*) promoter driving a *FIREFLY LUCIFERASE* (*fLUC*) reporter gene is transactivated by MYC2 in tobacco (*Nicotiana tabacum*) protoplasts (Pauwels et al., 2008). Cotransfecting JAZ12 with MYC2 abolished this induction completely (Fig. 1E). This repressive effect of *JAZ12* coexpression was dose dependent, as illustrated by a titration curve using decreasing amounts of JAZ12 (Fig. 1F).

Finally, we isolated a transfer DNA (T-DNA) insertion line in *JAZ12*, which we called *jaz12-1*. The T-DNA is inserted in the Jas intron (Chung and Howe, 2009; Supplemental Fig. S1A). Therefore, we tested the expression of *JAZ12* with primers 5′ and 3′ of the T-DNA.

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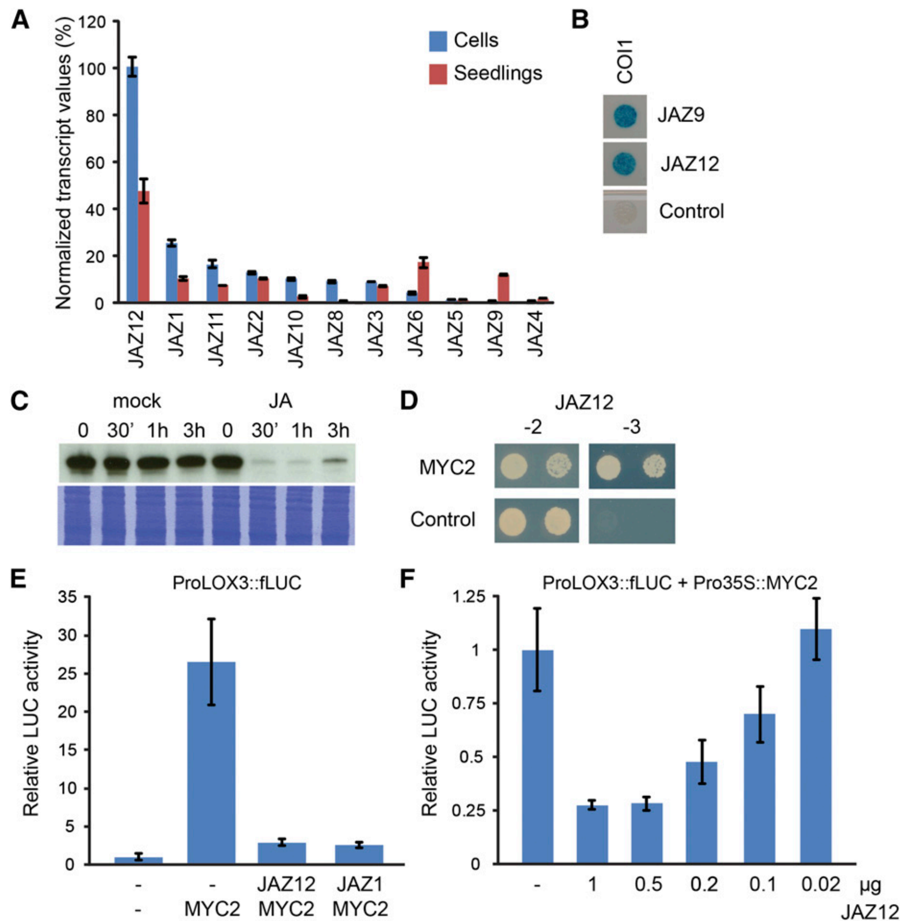

Figure 1. JAZ12 is a prototypal JAZ protein. A, *JAZ12* is a highly expressed *JAZ* gene in Arabidopsis suspension cultured cells and seedlings. Reads per kilobase per million mapped for each JAZ gene were plotted relative to *JAZ12* using RNA-Seq data from Arabidopsis suspension cultured cells (blue) and seedlings (red). *JAZ7* transcripts were not detected. B, COR mediates a direct interaction between COI1 and JAZ12. The EGY48 (p8opLacZ) yeast (*Saccharomyces cerevisiae*) strain was cotransformed with COI1 in pGILDA and JAZ9 and JAZ12 in pB42AD or pB42AD alone (control). Transformed yeasts were spotted on inducing medium containing Gal and raffinose supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid and 50 μM COR. C, JAZ12 is rapidly degraded by JA. Seven-day-old cell cultures expressing *JAZ12-GS* were treated with 50 μM JA or an equal volume of ethanol for the time points indicated. JAZ12-GS was detected by immunoblot using peroxidase anti-peroxidase (top), and the membrane was stained to inspect equal loading with Coomassie Blue (bottom). D, JAZ12 interacts directly with MYC2. The PJ69-4A yeast strain was cotransformed with JAZ12 in pGBKT7 and MYC2 in pGAD424gate or pGAD424gate as a control. The 10× and 100× dilutions of transformed yeasts were spotted on control medium lacking Leu and Trp (-2) or selective medium additionally lacking His (-3). E, JAZ12 is capable of repressing MYC2-mediated transactivation of the *LOX3* promoter. Tobacco protoplasts were transfected with a pLOX3-fLUC reporter construct and the indicated effector constructs in p2GW7, resulting in Pro-35S-mediated overexpression of *MYC2*, *JAZ12/JAZ1*, or both. A construct with the *Renilla reniformis* LUC (rLUC) under control of Pro-35*S* was cotransfected for the normalization of fLUC activity. Error bars represent SE of eight biological replicates. Two micrograms of each construct was transfected, and total DNA added was equalized with p2GW7-GUS plasmid. F, Dose-dependent inhibition of MYC2 activity by JAZ12. The experiment was as in E, but p2GW7-JAZ12 was transfected at the indicated amounts of DNA, equalized for total plasmid DNA with p2GW7-GUS.

While *JAZ12* expression 5′ of the T-DNA was only modestly (approximately 50%) reduced (Supplemental Fig. S1B), very few transcript 3′ of the T-DNA (approximately 10%) could be found (Supplemental Fig. S1B). JA-induced root growth inhibition in *jaz12-1* plants was similar to that in control plants (Supplemental Fig. S1C). This suggests that either enough functional JAZ12 protein is present in this line or that redundancy exists with other JAZ proteins. The latter is in line with a previous report that single *jaz* loss-of-function mutants lack a phenotype (Thines et al., 2007).

KEG Interacts Specifically with JAZ12

In the TAP analysis, we identified KEG as an interactor of JAZ12 (Table I). In parallel, we performed a Y2H library screen using full-length KEG as bait. This

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screen identified JAZ12 as a direct interactor. Out of the 40 colonies with in-frame interactors, 35 corresponded to JAZ12. The other five interactors were FATTY ACID BIOSYNTHESIS2 (At2g43710), CHLOROPHYLL *a*/*b*-BINDING PROTEIN2, GLUTAMATE RECEPTOR3.4 (At1g05200), chlorophyll-binding PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE2, and an oxidoreductase (At1g60710), all picked up only once in the Y2H screen. Subcloning of *KEG* revealed that the Y2H interaction with JAZ12 was mediated by the C-terminal HERC2-like repeat domain of KEG (Fig. 2A). Conversely, full-length KEG, as well as the fragment consisting of only the HERC2-like repeat domain, interacted specifically with the Jas domain of JAZ12 (Fig. 2B). MYC2 and NINJA were used as positive controls for interactions with the Jas and ZIM domains, respectively.

We also cloned the *JAZ12-1* variant from *jaz12-1* complementary DNA. The T-DNA insertion in the Jas intron in this line leads to the expression of an mRNA with the Jas intron retained. Because a stop codon is present in the intron, it results in a JAZ12 variant with a partial Jas domain, lacking C terminally the PY sequence (Supplemental Fig. S1A). As expected, the COR-dependent interaction of JAZ12-1 with COI1 could no longer be observed (Fig. 2C). As controls, we included JAZ12(RR142/143AA) and JAZ12(F149A) constructs in which residues of the Jas motif that are essential for COI1 interaction were mutated. These mutant JAZ12 proteins, however, were still capable of KEG interaction

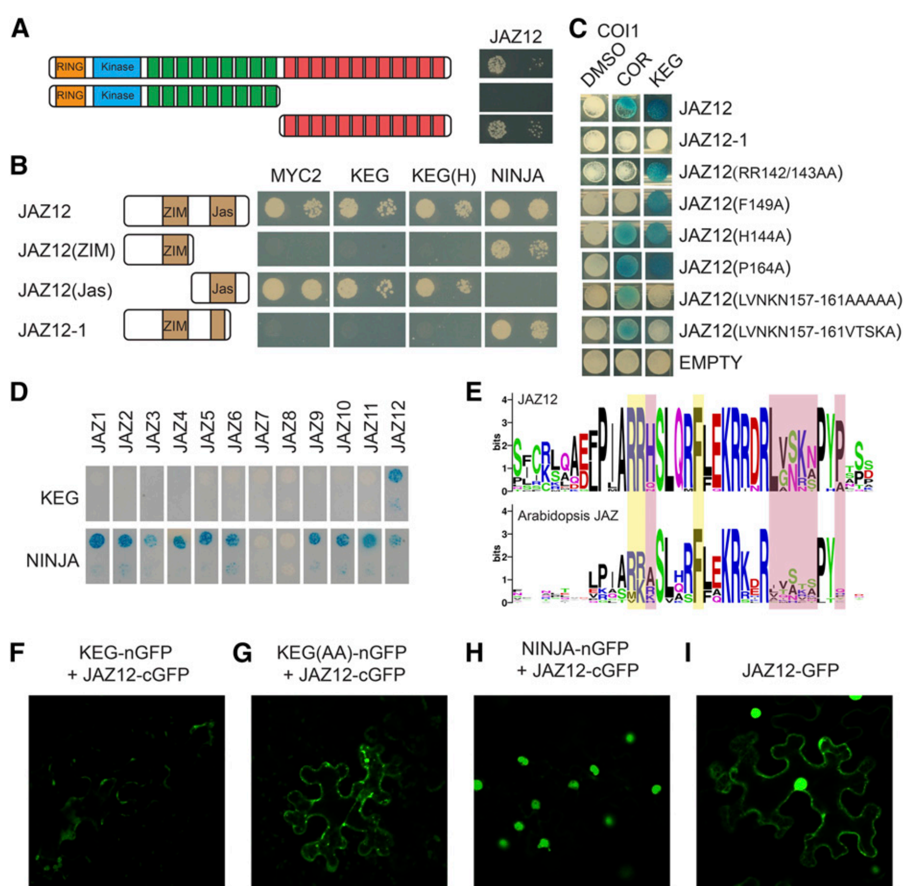

Figure 2. KEG interacts directly and specifically with JAZ12. A, JAZ12 interacts directly with the HERC2 repeats of KEG. The KEG protein structure is schematically represented with the RING domain in orange, kinase domain in blue, ankyrin repeats in green, and HERC2 repeats in red. B, The Jas domain of JAZ12 interacts with KEG. In A and B, the PJ69-4A yeast strain was cotransformed with JAZ12 in pGBKT7 and MYC2, NINJA, KEG, or KEG fragments in pGADT7gate. The 10× and 100× dilutions of transformed yeasts were spotted on selective medium lacking His. C, Interaction between KEG or COI1 and JAZ12, JAZ12-1, or other JAZ12 mutants. D, JAZ12 is the only JAZ protein capable of interacting with KEG. In C and D, the EGY48 (p8opLacZ) yeast strain was cotransformed with KEG or NINJA in pGILDA and JAZ in pB42AD. Transformed yeasts were spotted on inducing medium containing Gal and raffinose supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid and 50 μM COR or dimethyl sulfoxide (DMSO) as indicated. E, WebLogo consensus sequence of the Jas motif in JAZ12 orthologs (top) and Arabidopsis JAZ proteins (bottom). Amino acids essential for COI1 interaction are highlighted in yellow and those assayed for KEG interaction in pink. F to I, KEG interacts extranuclearly with JAZ12. F and G, *N. benthamiana* leaf epidermal cells were transiently transformed with JAZ12-cGFP and KEG-nGFP (F) or KEG(AA)-nGFP (G). H, NINJA-nGFP interacts in the nucleus with JAZ12-cGFP. I, JAZ12-GFP localizes to both the nucleus and the cytoplasm in transiently transformed *N. benthamiana* leaves.

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(Fig. 2C; Sheard et al., 2010). It has been shown previously that ΔPY variants of JAZ2.2 and JAZ10.3 can still interact with MYC2 (Chung and Howe, 2009). In contrast, JAZ12-1 did not interact with MYC2 or with KEG but still interacted with NINJA (Fig. 2B).

As the Jas domain is conserved in all JAZ proteins, we next tested all 12 JAZ proteins for interaction with KEG using Y2H assays. Interestingly, only JAZ12 was capable of interacting with KEG, both in the LexA-based (Fig. 2D) and the GAL4-based (Supplemental Fig. S2A) systems. NINJA was used as a positive control in these assays, as it interacts with most JAZ proteins (Fig. 2D). As KEG is known to autoubiquitinate in vitro, causing its degradation (Liu and Stone, 2010), we hypothesized that this might prevent interactions in yeast. Therefore, we also tested the KEG(AA) variant, which has a disrupted RING domain (C29A, H31A, and C34A; Stone et al., 2006) and can no longer autoubiquitinate, for interaction with all JAZ proteins. We again observed interaction only with JAZ12 (Supplemental Fig. S2B).

To identify what makes JAZ12 unique, we identified JAZ12 orthologs in different plant species based on synteny (Supplemental Fig. S2C). Several amino acids in the JAZ12 Jas domain were more conserved between JAZ12 orthologs as compared with other JAZ proteins in Arabidopsis (Fig. 2E; Supplemental Fig. S2D). Based on this comparison, we changed His-144, Pro-164, or a stretch of five amino acids, LVNKN157-161, to Ala(s) and tested the interaction with COI1 and KEG. With the latter construct, KEG interaction was lost while the COR-mediated COI1 interaction was retained (Fig. 2C). Finally, we changed the sequence LVNKN with the corresponding sequence of JAZ1, VTSKA. Again, KEG interaction was lost, suggesting that one or more of these amino acids that are conserved between JAZ12 orthologs are necessary for KEG interaction.

Finally, we assessed the JAZ12-KEG interaction using bimolecular fluorescence complementation (BiFC) by fusing the proteins with N-terminal or C-terminal fragments of GFP (designated nGFP and cGFP, respectively). When wild-type *KEG-nGFP* and *JAZ12-cGFP* were transiently coexpressed together in *Nicotiana benthamiana* leaves, we observed only a weak GFP signal, which was detected only outside the nucleus (Fig. 2F). However, when we used KEG(AA)-nGFP, a strong signal in the cytoplasm was observed (Fig. 2G). This corresponds to the reported extranuclear localization of KEG-GFP (Gu and Innes, 2011) and the localization of the interaction between KEG and ENHANCED DISEASE RESISTANCE1 (EDR1; Gu and Innes, 2012) and between KEG and ABI5 (Liu and Stone, 2013). Coexpression of *NINJA-nGFP* led to a nuclear signal (Fig. 2H). Correspondingly, the expression of *JAZ12-GFP* resulted in expression in both the nucleus and the cytoplasm in *N. benthamiana* leaves (Fig. 2I). However, the roots of a stable 35S cauliflower mosaic virus promoter-driven JAZ12-GFP Arabidopsis line only expressed detectable *GFP* in the nucleus (Supplemental Fig. S2, E and F).

Table II. *Overview of the proteins purified by TAP with GS-KEG(AA)*

Information is as in Table I. Numbers indicates the times the prey was identified in two experiments per column. AGI, Arabidopsis Genome Initiative identifier; CIPK26, CALCINEURIN B-LIKE PROTEIN-INTERACTING PROTEIN KINASE26; EIF6A, EUKARYOTIC INITIATION FACTOR6A; SDE3, SILENCING DEFECTIVE3. –, Prey was not identified in this experiment.

AGI	Protein	−JA	+JA
AT5G13530	KEG	2	2
AT1G05460	SDE3	–	2
AT3G55620	EIF6A	–	2
AT5G21326	CIPK26	–	2

TAP of KEG(AA)

To assess the KEG interactome further, we generated Arabidopsis cells expressing N-terminal GS-tagged *KEG*(*AA*) for TAP assays (Table II; Supplemental Data Set S1). By using the *KEG*(*AA*) mutant, we hoped to stabilize the tagged KEG protein and its targets, because ubiquitination and subsequent degradation are prevented. We treated the cells with 50 μM JA for 1 min. Although we could not find peptides for JAZ12 or ABI5/ABFs, we did find peptides of the known KEG target protein CIPK26, a calcineurin B-like interacting protein kinase (Lyzenga et al., 2013).

KEG Knockdown Lines Are ABA Hypersensitive

All three isolated knockout mutants of *KEG*, *keg1*, *keg2*, and *keg3*, are seedling lethal, leading to postgerminative growth arrest (Stone et al., 2006). To allow physiological analysis of KEG function, we used the artificial microRNA (amiRNA) method (Schwab et al., 2006) to generate a knockdown line of *KEG*. We obtained a single *KEG* amiRNA line (line 14) that showed normal Mendelian inheritance and for which we could select a homozygous population. This line showed retarded growth (Fig. 3A), although it had only modestly (±50%) reduced *KEG* expression at the RNA level (Fig. 3D). The effect on KEG protein levels could be more dramatic because it has been reported that, in plants, amiRNAs often do not only change transcript levels but repress translation, leading to reduced protein levels (Li et al., 2013). To evaluate the success of *KEG* knockdown, we analyzed the reported ABA hypersensitivity of *KEG* knockout mutants (Stone et al., 2006). The amiRNA line had a stronger reduction of primary root growth than wild-type Columbia-0 (Col-0) when grown on 5 μM ABA (Fig. 3, B and C) but not on 2.5 μM JA. Moreover, *KEG* amiRNA seedlings grown on control medium had a severely reduced number of lateral roots after 11 d (Fig. 3B). Inhibition of lateral root biogenesis is a well-known effect of exogenous ABA treatment (De Smet et al., 2003). We then checked the expression of several ABA and JA marker genes in this line (Fig. 3D). The ABA markers *RESPONSIVE TO ABSCISIC ACID18* (*RAB18*) and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) were clearly up-regulated.

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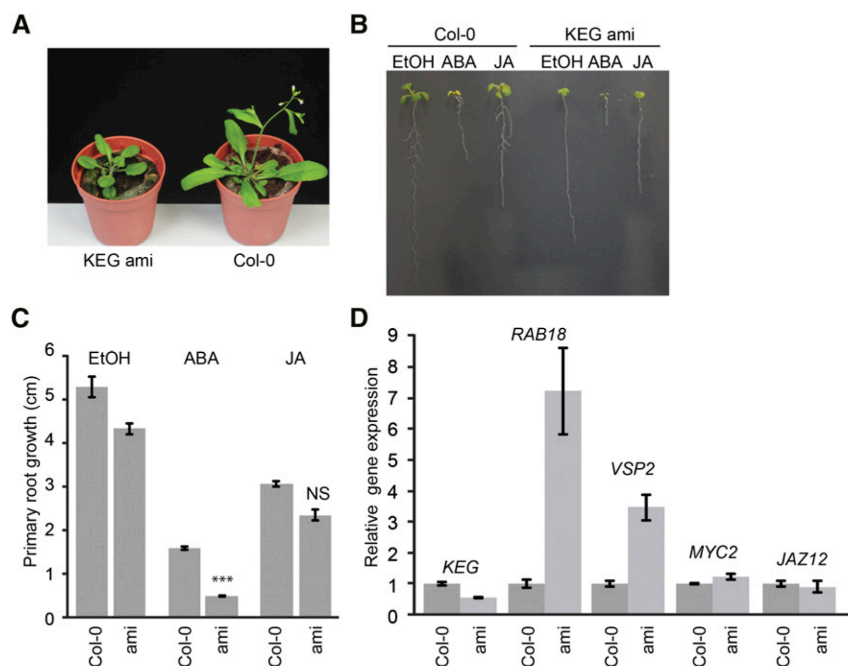

Figure 3. *KEG* knockdown lines are hypersensitive to ABA. A, Retarded growth of soil-grown *KEG* amiRNA line 14 plants. B and C, Root growth assays. Plants were grown for 4 d and transferred to medium containing ethanol (EtOH; control), 5 μM ABA, or 2.5 μM JA for 6 d. B, Representative seedlings. C, Average increase in primary root length after transfer. Values indicate means of three biological repeats (each consisting of eight seedlings) $\pm$ SE. The treatment $\times$ genotype interaction effect (two-way ANOVA) is indicated (***, $P < 0.001$). NS, Not significant. There were also significant effects ($P < 0.001$) of each treatment and of the genotype. D, Expression of *KEG*, *RAB18*, *VSP2*, *MYC2*, and *JAZ12* in ethanol control seedlings from B. Error bars depict SE ($n = 3$).

Although *VSP2* is a known JA marker, *VSP2* expression is known to be up-regulated by ABA as well (Anderson et al., 2004). The JA markers *MYC2* and *JAZ12*, on the other hand, were not induced (Fig. 3D). Based on these data, we conclude that *KEG* amiRNA lines have a constitutive ABA response in the absence of exogenous ABA.

The amiRNA targeting *KEG* was expressed under the control of a 35S promoter using the pFAST-R02 vector (Shimada et al., 2010). This vector harbors a red fluorescent protein (RFP) fusion expressed in the dormant dry seed, facilitating the selection of transformants. We used this property to confirm the phenotype of line 14 using a second independent transformant, line 2, which showed non-Mendelian inheritance and for which, even after several generations, no homozygous line was obtained. We used the presence of the RFP marker to our advantage and compared RFP− with RFP+ plants in experiments with this line. RFP+ plants showed reduced *KEG* levels (Supplemental Fig. S3A) and ABA hypersensitivity (Supplemental Fig. S3, B and C).

KEG Is a Positive Regulator of JAZ12 Stability

We used the *KEG* amiRNA lines also to test the relation between KEG activity and JAZ12 protein levels. Therefore, we crossed them with a *JAZ12-GFP* line and tested JAZ12-GFP levels by immunoblot analysis (Fig. 4A). To our surprise, JAZ12-GFP levels were decreased in the *KEG* knockdown lines (Fig. 4A; Supplemental Fig. S4A), whereas the transcript levels of the *JAZ12-GFP* transgene were unaffected (Supplemental Fig. S4B).

KEG is known to autoubiquitinate in the presence of ABA, leading to its degradation (Liu and Stone, 2010). Therefore, we treated seedlings producing GS-tagged JAZ12 with ABA by transferring them to liquid Murashige and Skoog (MS) medium containing ABA or ethanol. After transfer, JAZ12-GS is degraded over time in the control, and the addition of ABA results in increased JAZ12 degradation (Fig. 4B), in a dose-dependent manner (Fig. 4C). After 6 h, we observed a nearly complete loss of JAZ12-GS, while GS-tagged JAZ1, JAZ10, or the unrelated AUXIN SIGNALING F-BOX2 (AFB2) did not show alteration in protein levels compared with mock treatment (Fig. 4D). HA-tagged JAZ9 protein levels also showed some reduction after ABA treatment, but to a far lesser extent than JAZ12-GS (Fig. 4E).

To study the effect of *KEG* overexpression on JAZ12 stability further, we used transient expression in *N. benthamiana*. Whereas the expression of *JAZ12-GS* alone resulted in strong protein accumulation, coexpressing *COI1* abolished JAZ12-GS levels completely (Fig. 5A). When coexpressing *KEG* with *JAZ12-GS*, we did not observe changes in the JAZ12-GS levels, compared with the *JAZ12-GS* expressed in the absence of *KEG* (Fig. 5A). However, when coexpressing *KEG* together with *COI1* and *JAZ12-GS*, we could observe a protective effect of KEG on COI1-mediated JAZ12-GS degradation (Fig. 5A). Finally, we transiently expressed *JAZ12* fused to *GFP* together with *COI1* and/or *KEG* to study if COI1-mediated degradation and protection by KEG were specific for a subcellular compartment. This assay indicated that JAZ12-GFP was degraded both inside and outside the nucleus in the presence of COI1. Similarly, coexpression of *KEG* restored JAZ12 levels both intranuclearly and extranuclearly (Fig. 5B). Taken together, our data demonstrate that nucleus-localized COI1 and TGN-localized KEG both influence JAZ12 levels independently of JAZ12 subcellular localization.

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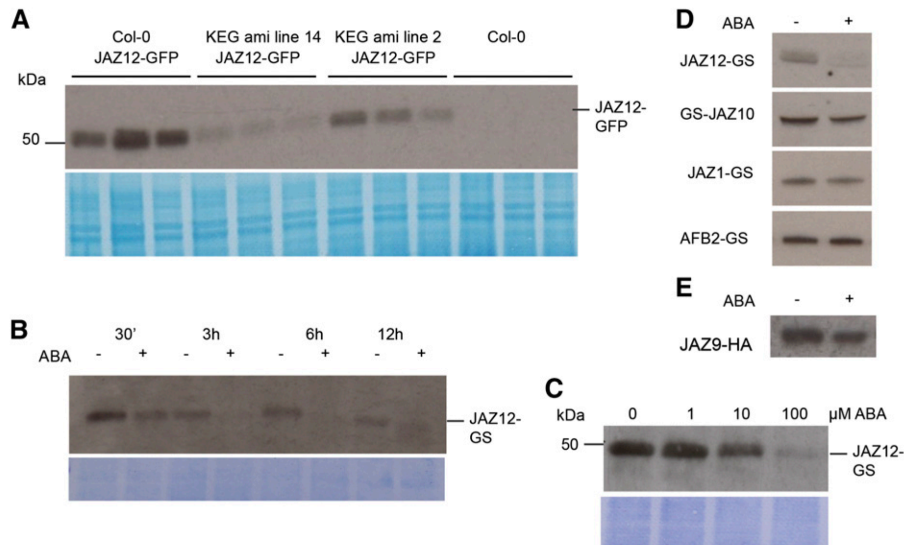

Figure 4. KEG is a positive regulator of JAZ12 stability. A, Immunoblot showing JAZ12-GFP levels in three biological repeats of a *JAZ12-GFP* line, its crosses with the *KEG* amiRNA lines, and the wild type (Col-0). Crosses were homozygous for *JAZ12-GFP* and RFP+, the latter indicative of *KEG* amiRNA expression. B, ABA enhances JAZ12-GS degradation. Seedlings were transferred from agar to liquid MS medium supplemented with 100 μM ABA (+) or ethanol (−), and protein levels were monitored over time. C, Dose response of ABA on JAZ12-GS protein levels 6 h after transfer. Membranes were stained with Coomassie Blue to inspect equal loading. D and E, ABA-mediated degradation of other JAZ proteins and AFB2. Immunoblots show the expression of GS-tagged (D) or hemagglutinin (HA)-tagged (E) proteins treated with ethanol or 100 μM ABA for 6 h. A representative experiment of three biological repeats is shown. In all experiments, 6-d-old seedlings were used.

DISCUSSION

Use of TAP-Mass Spectrometry to Study Ubiquitin E3 Ligases

Although the interaction between SCF^{COI1} and JAZ proteins is well established and has been studied using methods such as Y2H (Thines et al., 2007; Melotto et al., 2008) and pull-down (Thines et al., 2007; Fonseca et al., 2009) assays, we show here that the COI1-JAZ interaction can be determined in vivo using TAP-mass spectrometry using either the F-box protein or its target as bait. In our cell culture system, the interaction was dependent on the addition of JA, which presumably is converted to the bioactive JA-Ile form in vivo. In seedling cultures, however, the interaction was already observed without any exogenous JA treatment. Accordingly, in this system, we were able to detect tryptic peptides of JAZ12, which had the di-Gly mark of ubiquitination. Previously, direct proof of the ubiquitination of JAZ proteins was scarce. To our knowledge, such in vivo proof of ubiquitination has

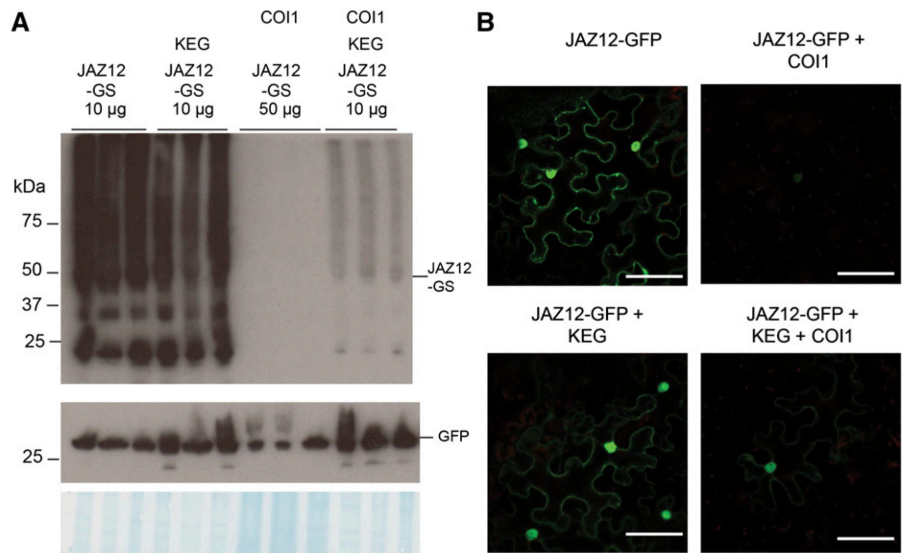

Figure 5. KEG and COI1 influence JAZ12-GFP levels independent of its subcellular localization. A, Immunoblot showing JAZ12-GS and GFP levels in *N. benthamiana* leaves coinfiltrated with *JAZ12-GS*, *KEG*, and/or *COI1*. *GFP* was cotransformed as a control. Each combination was coinfiltrated in triplicate. COI1-containing protein extracts were loaded in 5× excess (50 μg). The bottom gel shows Coomassie Blue staining of the blotted membrane. B, *N. benthamiana* leaves were transiently transformed with *JAZ12-GFP*, *COI1*, and/or *KEG* and imaged using a confocal microscope. Bars = 50 μm.

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only been reported for JAZ6 via an untargeted proteomics screen for ubiquitinated proteins (Saracco et al., 2009). Our TAP-mass spectrometry approach also allowed us to identify core components of the SCF complex (ASK1, ASK2, and CUL1) using either the F-box protein or its target JAZ12. This confirms the association of COI1 with an SCF complex (Xu et al., 2002). Recently, it was shown that the association with ASK1 and CUL1 is essential for COI1 stability (Yan et al., 2013).

Notably, we detected the interaction of JAZ12 only with MYC2, MYC3, and MYC4, both in cell cultures and in seedlings, although many other transcription factors have been reported to interact with the JAZ proteins, including JAZ12 (Pauwels and Goossens, 2011; Hu et al., 2013; Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013; Fonseca et al., 2014). Similarly, JAZ12 is the only JAZ for which we observed interaction using COI1 as bait. Negative results using TAP-mass spectrometry can be related to the expression levels of target proteins, and indeed, *JAZ12* was found to be a highly expressed *JAZ*, at least at the transcript level.

JAZ12 Interacts with KEG in the Cytoplasm

TAP with JAZ12 identified the E3 ligase KEG as an interactor of JAZ12. KEG is known to play a role in ABA signaling and interacts with the bZIP transcription factors ABI5, ABF1, and ABF3 through the ankyrin-repeat domain and with the kinase CIPK26 (Stone et al., 2006; Chen et al., 2013; Lyzenga et al., 2013). KEG promotes the proteasome-mediated degradation of these proteins in order to repress ABA signaling.

KEG also plays a role in pathogen resistance. The kinase EDR1 interacts at the TGN with KEG through the HERC2-like repeat domain. The *keg4* allele carrying a missense mutation in this domain was identified to suppress the *edr1*-mediated increased resistance to *Golovinomyces cichoracearum* (Wawrzynska et al., 2008). The *keg4* mutation or deletion of the entire HERC2-like repeat domain led to a primarily cytoplasmic localization of KEG, while this was unobserved for wild-type KEG (Gu and Innes, 2011).

The localization of KEG to the TGN and the activity in the nucleus by association with transcription factors could be reconciled by the finding that, although ABI5 is observed to be exclusively nuclear, the interaction between KEG and ABI5 localized to the cytoplasm when the RING domain of KEG was inactivated or a conserved Lys in ABI5 was mutated (Liu and Stone, 2013). Cytoplasmic turnover of ABI5 by KEG is proposed to also control nuclear ABI5 levels and, hence, its activity (Liu and Stone, 2013).

Here, we found JAZ12-GFP to be exclusively in the nucleus in Arabidopsis seedling roots, but upon transient overexpression in *N. benthamiana* leaves, it was also seen in the cytoplasm. As with ABI5, we found that the interaction between KEG and JAZ12 occurs outside the nucleus. For JAZ9, it was shown that nuclear localization is dependent on the interaction with the MYC2 transcription factor, and YFP-tagged JAZ9 accumulated in the cytosol in a *myc2* mutant (Withers et al., 2012). MYC2, MYC3, and MYC4 are known to be short-lived proteins regulated by proteasomal degradation (Zhai et al., 2013; Chico et al., 2014). GFP-tagged MYC2 is largely removed from the cell in the dark and far-red light (Chico et al., 2014), and MYC2 protein levels oscillate over time under the control of the circadian clock (Shin et al., 2012).

Determination of the detailed physiological and spatiotemporal circumstances in which JAZ12, possibly through (absence of) MYC2 interaction, favors interaction with KEG outside the nucleus will be the subject of further study.

Toward a Functional Role for the KEG-JAZ12 Interaction

We show here that JAZ12 is ubiquitinated in vivo, is a target of the SCF^{COI1} complex, and is degraded upon a JA stimulus, thus behaving like a canonical JAZ protein. Signal transducers are commonly targeted by multiple ubiquitin E3 ligases. ABI5, for example, is also targeted by the CUL4-based E3 ligases ABSCISIC ACID-HYPERSENSITIVE DAMAGED DNA BINDING PROTEIN1 (DDB1)-CULLIN4–associated factor1 (ABD1) and DDB1 binding WD40 HYPERSENSITIVE TO ABSCISIC ACID2 (DWA1)/DWA2 besides KEG (Lee et al., 2010; Seo et al., 2014). Unexpectedly, however, our results did not support our intuitive hypothesis of KEG as an instigator of JAZ12 ubiquitination and subsequent degradation. On the contrary, several observations pointed to KEG as a positive regulator of JAZ12 stability: (1) a *KEG* amiRNA line showed reduced JAZ12-GFP levels; (2) *KEG* overexpression protected JAZ12 from COI1-mediated degradation; and (3) ABA promoted JAZ12 degradation.

We considered the possibility that these observations are independent of the interaction between KEG and JAZ12 and caused by the repression of ABA signaling by KEG. The *KEG* amiRNA lines presented here indeed showed ABA hypersensitivity at the seedling stage, consistent with earlier reports studying loss-of-function *keg* alleles (Stone et al., 2006). ABA induces JA marker genes such as *VSP2* (Anderson et al., 2004), which is also induced in the *KEG* amiRNA line. Moreover, ABA biosynthesis is known to be required for the increase in JA levels after *Pythium irregulare* infection (Adie et al., 2007). Notwithstanding, we did not observe any induction of JA marker genes, such as *MYC2*, nor any hypersensitivity to JA in the *KEG* amiRNA line. Furthermore, ABA treatment only affected JAZ12 levels dramatically, while JAZ1 and JAZ10 were unaffected. JAZ9 levels also decreased, albeit far more modestly. The latter is in line with a recent report showing degradation of the Jas9-Venus reporter upon ABA treatment (Larrieu et al., 2015). Hence, the reduced JAZ12 stability after ABA treatment and in the *KEG* amiRNA line is unlikely to be caused by a general elevated JA signaling. Nevertheless, the regulation of JAZ12 stability by KEG is linked to SCF^{COI1}: *KEG* overexpression protected JAZ12 from COI1-mediated

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degradation, which was not caused by a tethering of JAZ12 outside the nucleus. GFP-tagged JAZ12 was observed in increasing amounts in both the cytoplasm and the nucleus when *KEG* was coexpressed with *COI1*. Hence, this work highlights KEG as a novel point of cross talk between the plant hormones ABA and JA.

MATERIALS AND METHODS

Molecular Cloning

The open reading frames (ORFs) of *AFB2* and *COI1* lacking a stop codon and *JAZ10* with a stop codon were PCR amplified with the primers listed in Supplemental Table S1 and cloned in a Gateway-compatible entry clone. For KEG(AA), we used the entry clone described previously (Stone et al., 2006). For TAP constructs, a MultiSite Gateway LR reaction was performed with destination vectors pKCTAP and pKNTAP for C- and N-terminal fusions, respectively. In both cases, ORFs were fused to the GS-TAP tag and put under the control of the 35S promoter. JAZ12-GFP was constructed by recombining a JAZ12 entry clone with pGWB5 (Mita et al., 1995). The *KEG* amiRNA construct was designed with the Web MicroRNA Designer (www.weigelworld.org) and constructed by PCR amplification with the primers listed in Supplemental Table S1 and pRS300 as template (Schwab et al., 2006). The PCR product, to which attB sites were added, was recombined with pDONR221 (Invitrogen) and then pFAST-R02 (Shimada et al., 2010) as the destination vector. BiFC constructs for Pro-35S:ORF-tag or Pro-35S:tag-ORF using the N- and C-terminal halves of enhanced GFP were constructed by multisite Gateway reactions using pK7m34GW, pH7m34GW (Karimi et al., 2005), or pH7m24GW as described (Boruc et al., 2010) combined with pDONR207 and pDONR221 entry clones of JAZ12, NINJA, KEG, and KEG(AA) (with or without stop codon).

Plant Material

The *jaz12-1* (SALK_055032) allele was obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003). Seedlings were PCR genotyped using a T-DNA and gene-specific primers (Supplemental Table S1). Amplicons were sequenced to confirm the location of the T-DNA.

For stable transformation in Arabidopsis (*Arabidopsis thaliana*), the *Agrobacterium tumefaciens* strain C58C1 (pMP90) was used to transform Col-0 plants by floral dip (Clough and Bent, 1998). Transformants were selected based on kanamycin for JAZ12-GS and by ProOLE1:OLE1-RFP expression in seeds for *KEG* amiRNA lines.

In Vitro Plant Growth

For all the experiments described with plants grown in vitro, Arabidopsis seedlings were sterilized by the chlorine gas method and sown on sterile plates containing the corresponding growth medium. Plates were kept in the dark at 4°C for 2 d for stratification, after which they were transferred to a growth room with 21°C temperature and a 16-h-light/8-h-dark regime. The day of the transfer was considered as 0 d after stratification.

TAP

Arabidopsis cell suspension cultures (PSB-D) were transformed without callus selection as described previously (Van Leene et al., 2008). For treatments, 50 μM JA (Duchefa) was added to the cell culture for 1 min before harvesting cells in liquid nitrogen. Affinity purification and liquid chromatography-tandem mass spectrometry analysis were as described (Van Leene et al., 2015). For TAP of JAZ12-GS from seedlings, a homozygous line containing a single T-DNA locus was identified and grown in liquid MS medium as described (Van Leene et al., 2015).

RNA-Seq

RNA was isolated from wild-type PSB-D cell cultures and 14-d-old Col-0 seedlings in three biological repeats using the RNeasy Plant Mini Kit (Qiagen) and DNase I treated (Promega). A Trueseq RNA-Seq library (Illumina) was compiled and sequenced as 50-bp single read using Illumina HiSeq 2000 technology at GATC Biotech. Read quality control, filtering, mapping to The Arabidopsis Information Resource 10 Arabidopsis genome, and read counting were carried out using the Galaxy portal running on an internal server (http://galaxyproject.org/). Sequences were filtered and trimmed with the Filter FASTQ v1 and FASTQ Quality Trimmer v1 tools, respectively, with default settings (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were subsequently mapped to The Arabidopsis Information Resource 10 version of the Arabidopsis genome using GSNAPv2 (Wu and Nacu, 2010), allowing a maximum of five mismatches. The reads that uniquely map to the genome were used for quantification on the gene level with htseq-count from the HTSeq python package (Anders et al., 2015). Reads per kilobase per million values were calculated for each *JAZ* gene in each sample.

Y2H Analysis

The Y2H screen was conducted using a custom library manufactured by Invitrogen using RNA isolated from a mixture of seedlings and rosette leaves from short-day-grown plants. The latter were collected from plants at multiple time points following infection with *Pseudomonas syringae* pv *tomato* strain DC3000. The library was cloned into the Invitrogen pDEST22 low-copy vector. A bait construct containing full-length *KEG* was cloned into pDEST32. Library screening was performed following the protocol provided by Invitrogen. For both GAL4- and LexA-based assays, cloning, yeast strains, culturing, transformation, and reporter assays were done as described (Cuéllar Pérez et al., 2013), except for the COI1 interaction assays. In those, 50 μM COR (Sigma-Aldrich) was added to the medium or dimethyl sulfoxide as a control and 5-μL yeast suspensions were dropped manually on small petri plates. Entry clones for JAZ and MYC proteins were generated as described previously (Pauwels et al., 2010; Fernández-Calvo et al., 2011). Truncations and mutants of *JAZ12* were made by PCR amplification using the primers listed in Supplemental Table S1. KEG truncations were cloned as described previously (Stone et al., 2006).

Transient Expression Assays

Cloning, tobacco (*Nicotiana tabacum*) BY-2 protoplast preparation, automated transfection, lysis, and luciferase measurements were carried out as described previously (Vanden Bossche et al., 2013). The ProLOX3:fLUC and Pro-35S:MYC2 constructs were generated as described (Pauwels et al., 2008). *JAZ12* was cloned in the plasmid p2GW7 for overexpression (Vanden Bossche et al., 2013).

JA Degradation Assay

Seven-day-old cells expressing *JAZ12-GS* were subcultured in individual flasks for 2 d and treated with 50 μM JA or ethanol at the different time points. Cells were harvested and snap frozen in liquid nitrogen.

ABA Degradation Assay

Seedlings expressing epitope-tagged proteins were grown in vitro in pools of 10 to 15 seedlings for 6 d after germination. Each pool was transferred to 1 mL of MS medium without agar on a 24-well plate containing the indicated concentration of ABA or an equal volume of ethanol. After incubation for the indicated time in the growth room, seedlings were harvested and snap frozen in liquid nitrogen.

Immunoblotting

Total protein was extracted using extraction buffer (25 mM Tris-HCl, pH 7.6, 15 mM $MgCl_2$, 150 mM NaCl, 15 mM *p*-nitrophenyl phosphate, 60 mM β-glycerophosphate, 0.1% Nonidet P-40, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μM E64, complete proteinase inhibitor [Roche], and 5% ethylene glycol), and the protein concentration was determined using the Bradford assay (Bio-Rad). Samples were denatured in Laemmli buffer, run on a 4% to 15% TGX gel (Bio-Rad) for 20 min at 300 V, and subsequently blotted on a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad). Antibodies used were peroxidase anti-peroxidase (P1291; Sigma-Aldrich), anti-HA (3F10; Roche), and anti-GFP (abcam290) antibodies. Chemiluminescent detection was performed with Western Bright ECL (Isogen; http://www.isogen-lifescience.com/).

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Root Growth Assays

For *jaz12-1*, seedlings were grown on MS medium plates (10 g L^{-1} Suc and 8 g L^{-1} agar, pH 5.7) containing 2.5 or 10 μM JA (Duchefa) or ethanol. Seedlings were grown vertically under the conditions described above. Plates were scanned 11 d after stratification, and primary root length was measured by means of the EZ-Rhizo software (Armengaud et al., 2009).

For *KEG* amiRNA lines, seedlings were grown as above on MS plates for 4 d and then transferred to new plates containing 2.5 μM JA (Duchefa), 5 μM ABA (Sigma-Aldrich), or ethanol. Primary root length was marked, and seedlings were grown for another 6 d before scanning. Primary root growth following transfer was measured by means of ImageJ software (http://imagej.nih.gov).

Gene Expression Analysis

Frozen plant material was ground in a Retsch MM300 mixer, and total RNA was extracted using the Qiagen RNeasy kit (http://www.qiagen.com/). An RNase-free DNase step was performed following the manufacturer's instructions for the preparation of RNA. Next, 1 μg of total RNA was used for complementary DNA synthesis with the iScript kit (Bio-Rad; http://www.bio-rad.com/). Quantitative reverse transcription-PCR was performed on a LightCycler 480 system (Roche; http://www.roche.com) using the Fast Start SYBR Green I PCR mix (Roche). At least three biological repeats and three technical repeats were used for each analysis. Expression data were normalized using two reference genes, UBIQUITIN-CONJUGATING ENZYME21 (UBC21; At5g25760) and PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2AA3; At1g13320). The primer sequences are provided in Supplemental Table S1.

Transient Expression in *Nicotiana benthamiana*

Wild-type *N. benthamiana* plants (3–4 weeks old) were used as a transient protein expression system in the BiFC and JAZ12 stability experiments. Constructs were transiently expressed by *A. tumefaciens*-mediated transient transformation of lower epidermal leaf cells as described previously (Boruc et al., 2010) using a modified buffer (10 mM $MgCl_2$ [1 M stock solution; Merck], 10 mM MES [0.5 M stock solution; Duchefa], and 100 μM acetosyringone [100 mM stock solution; Sigma-Aldrich]) and the addition of a *P19*-expressing *A. tumefaciens* strain to boost protein expression (Voinnet et al., 2003). All *A. tumefaciens* strains were grown for 2 d, diluted to an optical density of 1 in infiltration buffer, and incubated for 2 h at room temperature before mixing in a 1:1 ratio with other strains and injecting. For BiFC and JAZ12-GFP stability assays, lower epidermal cells were examined for fluorescence using confocal microscopy 3 d after infiltration. For immunoblots, infiltrated leaf tissue was harvested 3 d after infiltration and immediately frozen in liquid nitrogen.

Confocal Microscopy

For subcellular localization of JAZ12-GFP in Arabidopsis seedlings, seedlings were briefly incubated in propidium iodide (3 mg L^{-1}; Sigma-Aldrich) and subsequently washed and mounted in milliQ water. Fluorescence microscopy was performed with an Olympus FV10 ASW confocal microscope.

Bioinformatics

Orthologs of Arabidopsis JAZ12 were identified using the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication) based on synteny. Sequences were aligned using MUSCLE with default settings in JalView 2.8.2 (www.jalview.org). Protein sequences used for the alignment and alignment as a text file are available as Supplemental Data Set S2. Sequence logos were created using WebLogo (http://weblogo.berkeley.edu/).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Characterization of the *jaz12-1* T-DNA insertion line.

Supplemental Figure S2. KEG interacts specifically with JAZ12.

Supplemental Figure S3. *KEG* knockdown lines are hypersensitive to ABA.

Supplemental Figure S4. KEG is a positive regulator of JAZ12 stability.

Supplemental Table S1. Primers used in this study.

Supplemental Data Set S1. Mass spectrometry data from TAP experiments.

Supplemental Data Set S2. Protein sequences used for alignments.

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LITERATURE CITED

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell **15:** 63–78

Acosta IF, Gasperini D, Chételat A, Stolz S, Santuari L, Farmer EE (2013) Role of NINJA in root jasmonate signaling. Proc Natl Acad Sci USA **110:** 15473–15478

Adie BAT, Pérez-Pérez J, Pérez-Pérez MM, Godoy M, Sánchez-Serrano JJ, Schmelz EA, Solano R (2007) ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. Plant Cell **19:** 1665–1681

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301:** 653–657

Anders S, Pyl PT, Huber W (2015) HTSeq: a Python framework to work with high-throughput sequencing data. Bioinformatics **31:** 166–169

Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, Maclean DJ, Ebert PR, Kazan K (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. Plant Cell **16:** 3460–3479

Armengaud P, Zambaux K, Hills A, Sulpice R, Pattison RJ, Blatt MR, Amtmann A (2009) EZ-Rhizo: integrated software for the fast and accurate measurement of root system architecture. Plant J **57:** 945–956

Attaran E, Major IT, Cruz JA, Rosa BA, Koo AJ, Chen J, Kramer DM, He SY, Howe GA (2014) Temporal dynamics of growth and photosynthesis suppression in response to jasmonate signaling. Plant Physiol **165:** 1302–1314

Boruc J, Mylle E, Duda M, De Clercq R, Rombauts S, Geelen D, Hilson P, Inzé D, Van Damme D, Russinova E (2010) Systematic localization of the Arabidopsis core cell cycle proteins reveals novel cell division complexes. Plant Physiol **152:** 553–565

Chen YT, Liu H, Stone S, Callis J (2013) ABA and the ubiquitin E3 ligase KEEP ON GOING affect proteolysis of the Arabidopsis thaliana transcription factors ABF1 and ABF3. Plant J **75:** 965–976

Chico JM, Fernández-Barbero G, Chini A, Fernández-Calvo P, Díez-Díaz M, Solano R (2014) Repression of jasmonate-dependent defenses by shade involves differential regulation of protein stability of MYC transcription factors and their JAZ repressors in *Arabidopsis*. Plant Cell **26:** 1967–1980

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, et al (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448:** 666–671

Chung HS, Howe GA (2009) A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in *Arabidopsis*. Plant Cell **21:** 131–145

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16:** 735–743

Cuéllar Pérez A, Nagels Durand A, Vanden Bossche R, De Clercq R, Persiau G, Van Wees SCM, Pieterse CMJ, Gevaert K, De Jaeger G, Goossens A, et al (2014) The non-JAZ TIFY protein TIFY8 from Arabidopsis thaliana is a transcriptional repressor. PLoS One **9:** e84891

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Cuéllar Pérez A, Pauwels L, De Clercq R, Goossens A (2013) Yeast two-hybrid analysis of jasmonate signaling proteins. *In* A Goossens, L Pauwels, eds, Jasmonate Signal. Humana Press, Totowa, NJ, pp 173–186

De Smet I, Signora L, Beeckman T, Inzé D, Foyer CH, Zhang H (2003) An abscisic acid-sensitive checkpoint in lateral root development of Arabidopsis. Plant J **33:** 543–555

Fernández-Calvo P, Chini A, Fernández-Barbero G, Chico JM, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM, et al (2011) The *Arabidopsis* bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell **23:** 701–715

Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, Miersch O, Wasternack C, Solano R (2009) (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat Chem Biol **5:** 344–350

Fonseca S, Fernández-Calvo P, Fernández GM, Díez-Díaz M, Gimenez-Ibanez S, López-Vidriero I, Godoy M, Fernández-Barbero G, Van Leene J, De Jaeger G, et al (2014) bHLH003, bHLH013 and bHLH017 are new targets of JAZ repressors negatively regulating JA responses. PLoS One **9:** e86182

Garcia ME, Lynch T, Peeters J, Snowden C, Finkelstein R (2008) A small plant-specific protein family of ABI five binding proteins (AFPs) regulates stress response in germinating Arabidopsis seeds and seedlings. Plant Mol Biol **67:** 643–658

Goossens J, Swinnen G, Vanden Bossche R, Pauwels L, Goossens A (2015) Change of a conserved amino acid in the MYC2 and MYC3 transcription factors leads to release of JAZ repression and increased activity. New Phytol **206:** 1229–1237

Grunewald W, Vanholme B, Pauwels L, Plovie E, Inzé D, Gheysen G, Goossens A (2009) Expression of the Arabidopsis jasmonate signalling repressor JAZ1/TIFY10A is stimulated by auxin. EMBO Rep **10:** 923–928

Gu Y, Innes RW (2011) The KEEP ON GOING protein of Arabidopsis recruits the ENHANCED DISEASE RESISTANCE1 protein to trans-Golgi network/early endosome vesicles. Plant Physiol **155:** 1827–1838

Gu Y, Innes RW (2012) The KEEP ON GOING protein of *Arabidopsis* regulates intracellular protein trafficking and is degraded during fungal infection. Plant Cell **24:** 4717–4730

Hu Y, Jiang L, Wang F, Yu D (2013) Jasmonate regulates the INDUCER OF CBF EXPRESSION-C-REPEAT BINDING FACTOR/DRE BINDING FACTOR1 cascade and freezing tolerance in *Arabidopsis*. Plant Cell **25:** 2907–2924

Karimi M, De Meyer B, Hilson P (2005) Modular cloning in plant cells. Trends Plant Sci **10:** 103–105

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc Natl Acad Sci USA **105:** 7100–7105

Kazan K, Manners JM (2012) JAZ repressors and the orchestration of phytohormone crosstalk. Trends Plant Sci **17:** 22–31

Kirkpatrick DS, Denison C, Gygi SP (2005) Weighing in on ubiquitin: the expanding role of mass-spectrometry-based proteomics. Nat Cell Biol **7:** 750–757

Lackman P, González-Guzmán M, Tilleman S, Carqueijeiro I, Pérez AC, Moses T, Seo M, Kanno Y, Häkkinen ST, Van Montagu MCE, et al (2011) Jasmonate signaling involves the abscisic acid receptor PYL4 to regulate metabolic reprogramming in Arabidopsis and tobacco. Proc Natl Acad Sci USA **108:** 5891–5896

Larrieu A, Champion A, Legrand J, Lavenus J, Mast D, Brunoud G, Oh J, Guyomarc'h S, Pizot M, Farmer EE, et al (2015) A fluorescent hormone biosensor reveals the dynamics of jasmonate signalling in plants. Nat Commun **6:** 6043

Lee JH, Yoon HJ, Terzaghi W, Martinez C, Dai M, Li J, Byun MO, Deng XW (2010) DWA1 and DWA2, two *Arabidopsis* DWD protein components of CUL4-based E3 ligases, act together as negative regulators in ABA signal transduction. Plant Cell **22:** 1716–1732

Li JF, Chung HS, Niu Y, Bush J, McCormack M, Sheen J (2013) Comprehensive protein-based artificial microRNA screens for effective gene silencing in plants. Plant Cell **25:** 1507–1522

Liu H, Stone SL (2010) Abscisic acid increases *Arabidopsis* ABI5 transcription factor levels by promoting KEG E3 ligase self-ubiquitination and proteasomal degradation. Plant Cell **22:** 2630–2641

Liu H, Stone SL (2013) Cytoplasmic degradation of the Arabidopsis transcription factor abscisic acid insensitive 5 is mediated by the RING-type E3 ligase KEEP ON GOING. J Biol Chem **288:** 20267–20279

Lorenzo O, Chico JM, Sánchez-Serrano JJ, Solano R (2004) JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. Plant Cell **16:** 1938–1950

Lyzenga WJ, Liu H, Schofield A, Muise-Hennessey A, Stone SL (2013) Arabidopsis CIPK26 interacts with KEG, components of the ABA signalling network and is degraded by the ubiquitin-proteasome system. J Exp Bot **64:** 2779–2791

Melotto M, Mecey C, Niu Y, Chung HS, Katsir L, Yao J, Zeng W, Thines B, Staswick P, Browse J, et al (2008) A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. Plant J **55:** 979–988

Mita S, Suzuki-Fujii K, Nakamura K (1995) Sugar-inducible expression of a gene for β-amylase in *Arabidopsis thaliana*. Plant Physiol **107:** 895–904

Moreno JE, Shyu C, Campos ML, Patel LC, Chung HS, Yao J, He SY, Howe GA (2013) Negative feedback control of jasmonate signaling by an alternative splice variant of JAZ10. Plant Physiol **162:** 1006–1017

Nakata M, Mitsuda N, Herde M, Koo AJK, Moreno JE, Suzuki K, Howe GA, Ohme-Takagi M (2013) A bHLH-type transcription factor, ABA-INDUCIBLE BHLH-TYPE TRANSCRIPTION FACTOR/JA-ASSOCIATED MYC2-LIKE1, acts as a repressor to negatively regulate jasmonate signaling in *Arabidopsis*. Plant Cell **25:** 1641–1656

Niu Y, Figueroa P, Browse J (2011) Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in Arabidopsis. J Exp Bot **62:** 2143–2154

Pauwels L, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Pérez AC, Chico JM, Bossche RV, Sewell J, Gil E, et al (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature **464:** 788–791

Pauwels L, Goossens A (2011) The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell **23:** 3089–3100

Pauwels L, Morreel K, De Witte E, Lammertyn F, Van Montagu M, Boerjan W, Inzé D, Goossens A (2008) Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells. Proc Natl Acad Sci USA **105:** 1380–1385

Saracco SA, Hansson M, Scalf M, Walker JM, Smith LM, Vierstra RD (2009) Tandem affinity purification and mass spectrometric analysis of ubiquitylated proteins in Arabidopsis. Plant J **59:** 344–358

Sasaki-Sekimoto Y, Jikumaru Y, Obayashi T, Saito H, Masuda S, Kamiya Y, Ohta H, Shirasu K (2013) Basic helix-loop-helix transcription factors JASMONATE-ASSOCIATED MYC2-LIKE1 (JAM1), JAM2, and JAM3 are negative regulators of jasmonate responses in Arabidopsis. Plant Physiol **163:** 291–304

Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. Plant Cell **18:** 1121–1133

Seo KI, Lee JH, Nezames CD, Zhong S, Song E, Byun MO, Deng XW (2014) ABD1 is an *Arabidopsis* DCAF substrate receptor for CUL4-DDB1-based E3 ligases that acts as a negative regulator of abscisic acid signaling. Plant Cell **26:** 695–711

Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu FF, Sharon M, Browse J, et al (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature **468:** 400–405

Shimada TL, Shimada T, Hara-Nishimura I (2010) A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of Arabidopsis thaliana. Plant J **61:** 519–528

Shin J, Heidrich K, Sanchez-Villarreal A, Parker JE, Davis SJ (2012) TIME FOR COFFEE represses accumulation of the MYC2 transcription factor to provide time-of-day regulation of jasmonate signaling in *Arabidopsis*. Plant Cell **24:** 2470–2482

Shyu C, Figueroa P, Depew CL, Cooke TF, Sheard LB, Moreno JE, Katsir L, Zheng N, Browse J, Howe GA (2012) JAZ8 lacks a canonical degron and has an EAR motif that mediates transcriptional repression of jasmonate responses in *Arabidopsis*. Plant Cell **24:** 536–550

Song S, Qi T, Fan M, Zhang X, Gao H, Huang H, Wu D, Guo H, Xie D (2013) The bHLH subgroup IIId factors negatively regulate jasmonate-mediated plant defense and development. PLoS Genet **9:** e1003653

Stone SL, Williams LA, Farmer LM, Vierstra RD, Callis J (2006) KEEP ON GOING, a RING E3 ligase essential for *Arabidopsis* growth and development, is involved in abscisic acid signaling. Plant Cell **18:** 3415–3428

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature **448:** 661–665

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Thireault C, Shyu C, Yoshida Y, St Aubin B, Campos ML, Howe GA (2015) Repression of jasmonate signaling by a non-TIFY JAZ protein in Arabidopsis. Plant J **82:** 669–679

Vanden Bossche R, Demedts B, Vanderhaeghen R, Goossens A (2013) Transient expression assays in tobacco protoplasts. *In* A Goossens, L Pauwels, eds, Jasmonate Signal. Humana Press, Totowa, NJ, pp 227–239

Van Leene J, Eeckhout D, Cannoot B, De Winne N, Persiau G, Van De Slijke E, Vercruysse L, Dedecker M, Verkest A, Vandepoele K, et al (2015) An improved toolbox to unravel the plant cellular machinery by tandem affinity purification of Arabidopsis protein complexes. Nat Protoc **10:** 169–187

Van Leene J, Witters E, Inzé D, De Jaeger G (2008) Boosting tandem affinity purification of plant protein complexes. Trends Plant Sci **13:** 517–520

Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J **33:** 949–956

Wawrzynska A, Christiansen KM, Lan Y, Rodibaugh NL, Innes RW (2008) Powdery mildew resistance conferred by loss of the ENHANCED DISEASE RESISTANCE1 protein kinase is suppressed by a missense mutation in KEEP ON GOING, a regulator of abscisic acid signaling. Plant Physiol **148:** 1510–1522

Withers J, Yao J, Mecey C, Howe GA, Melotto M, He SY (2012) Transcription factor-dependent nuclear localization of a transcriptional repressor in jasmonate hormone signaling. Proc Natl Acad Sci USA **109:** 20148–20153

Wu TD, Nacu S (2010) Fast and SNP-tolerant detection of complex variants and splicing in short reads. Bioinformatics **26:** 873–881

Xu L, Liu F, Lechner E, Genschik P, Crosby WL, Ma H, Peng W, Huang D, Xie D (2002) The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in *Arabidopsis*. Plant Cell **14:** 1919–1935

Yan J, Li H, Li S, Yao R, Deng H, Xie Q, Xie D (2013) The *Arabidopsis* F-box protein CORONATINE INSENSITIVE1 is stabilized by SCF^{COI1} and degraded via the 26S proteasome pathway. Plant Cell **25:** 486–498

Yan Y, Stolz S, Chételat A, Reymond P, Pagni M, Dubugnon L, Farmer EE (2007) A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell **19:** 2470–2483

Zhai Q, Yan L, Tan D, Chen R, Sun J, Gao L, Dong MQ, Wang Y, Li C (2013) Phosphorylation-coupled proteolysis of the transcription factor MYC2 is important for jasmonate-signaled plant immunity. PLoS Genet **9:** e1003422

Zhu Z, An F, Feng Y, Li P, Xue L, A M, Jiang Z, Kim JM, To TK, Li W, et al (2011) Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in Arabidopsis. Proc Natl Acad Sci USA **108:** 12539–12544

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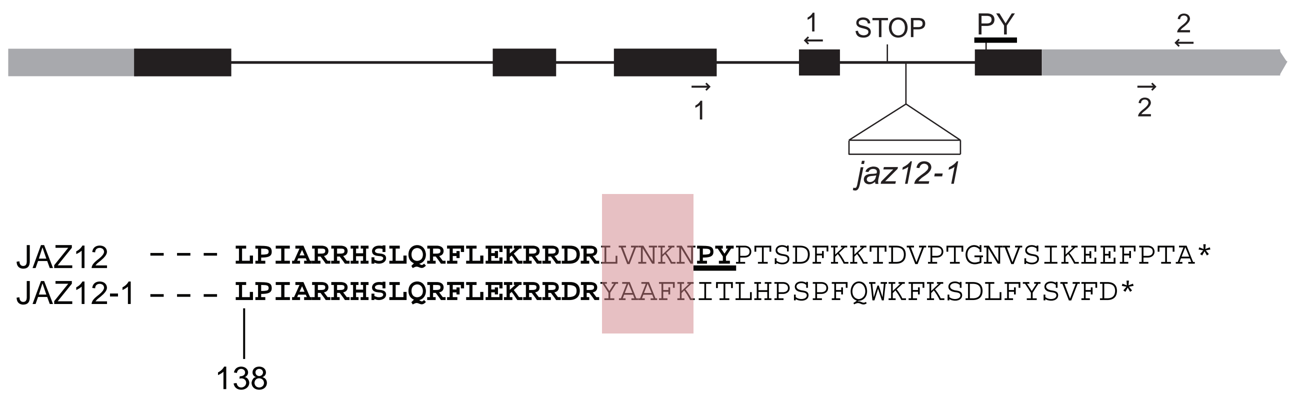
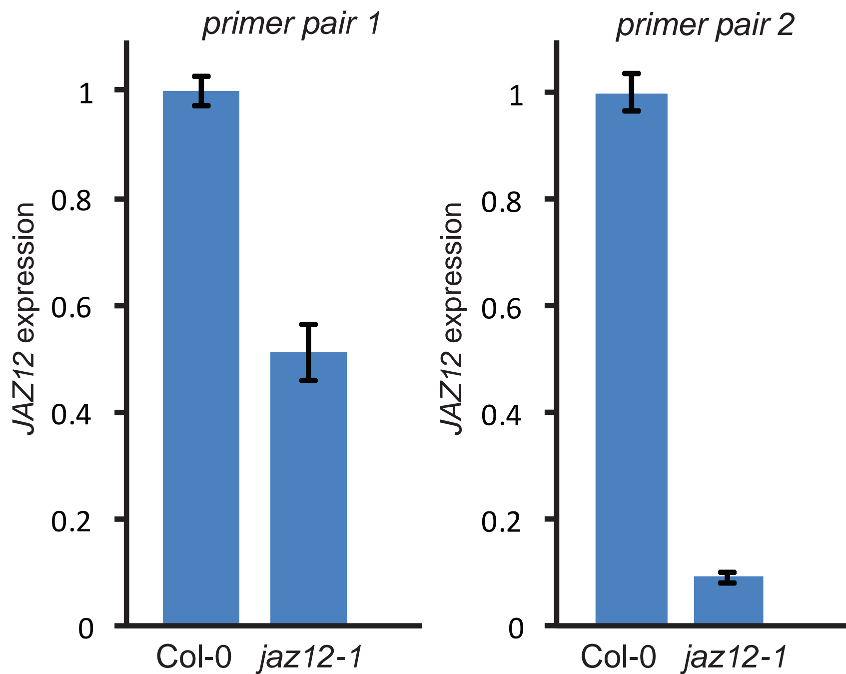




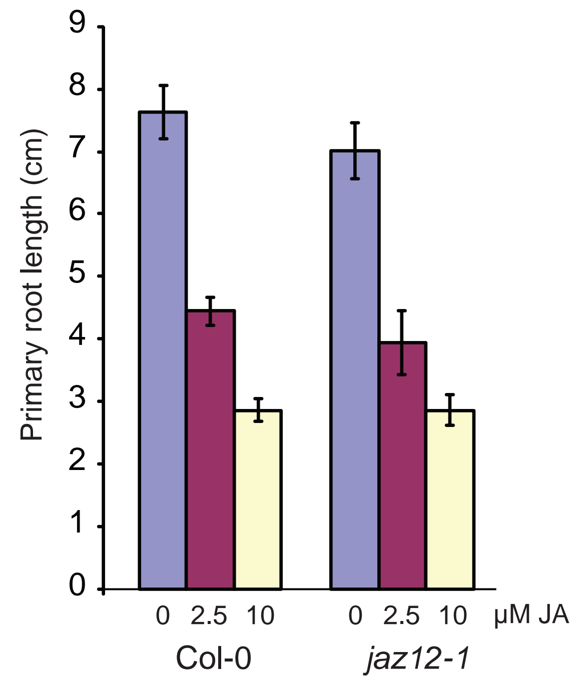


Figure S1. Characterization of the *jaz12-1* T-DNA insertion line. A, Schematic diagram of the *JAZ12* (AT5G20900) locus. Black bars, black lines and grey bars represent exons, introns and the untranslated regions, respectively. The T-DNA in the *jaz12-1* line (SALK_055032) is inserted in the Jas intron. The 5th exon contains the conserved PY motif. Arrows and numbers indicate different primer combinations covering different regions of *JAZ12*. Primer sequences can be found in Table SI. An alignment is shown between the WT JAZ12 and the JAZ12-1 amino acid sequences. While the N-terminal part of the Jas motif (in bold) is unaffected, the sequence essential for KEG interaction (highlighted in pink) and the PY motif (in bold underlined) are lost. B, RT-qPCR analysis of *JAZ12* expression in Col-0 and *jaz12-1* seedlings. Error bars represent SE of four biological replicates. C, Primary root length of Col-0 and *jaz12-1* seedlings grown on MS with 0, 2.5 or 10 µM JA. Error bars represent SE (12≤n<20). For B and C, seedlings were grown for 10 days in continuous light.

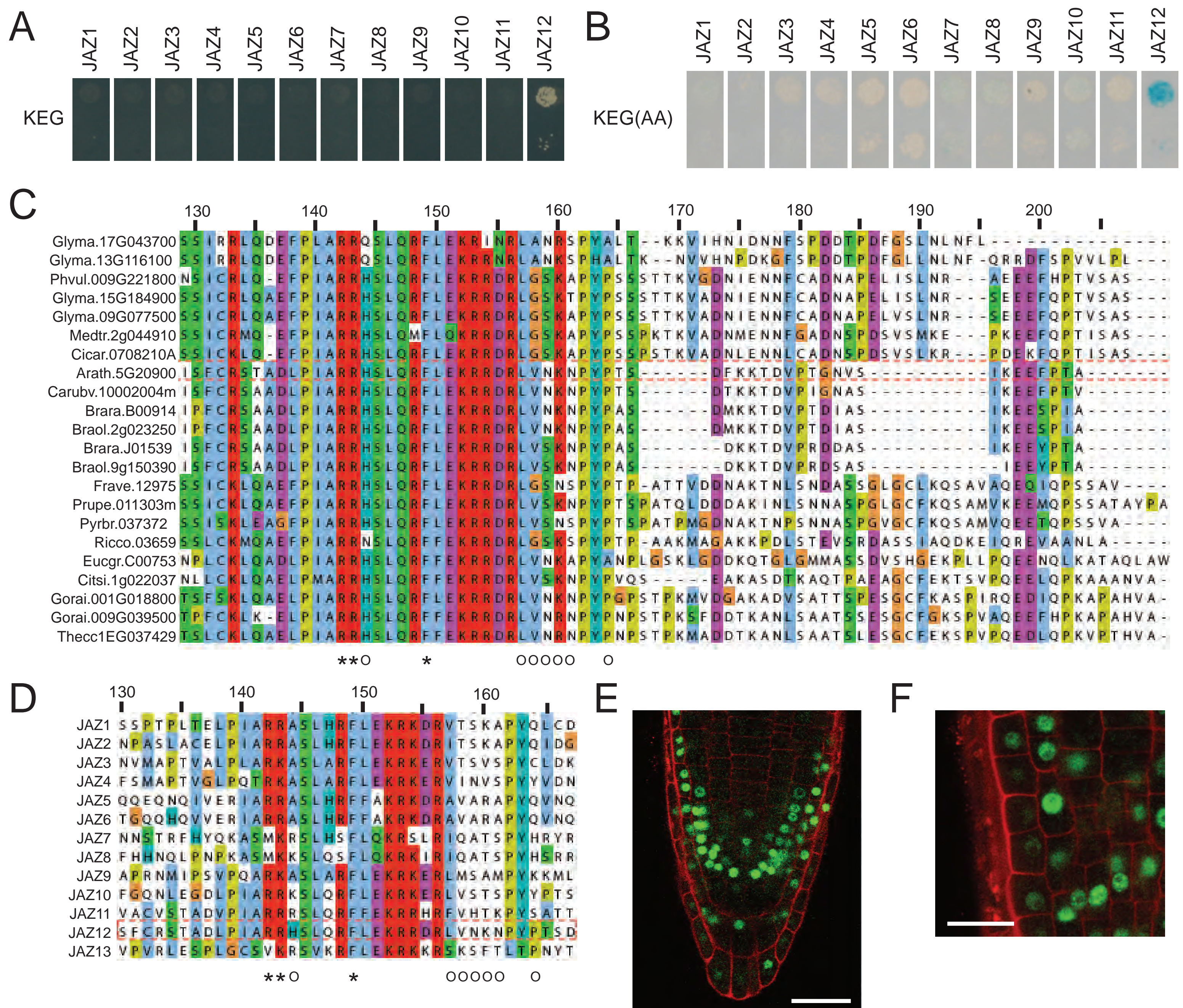


Figure S2. KEG interacts specifically with JAZ12. A, Only JAZ12 interacts directly with KEG in the GAL4-based Y2H system. The PJ69-4A yeast strain was co-transformed with JAZ proteins in pGBKT7 and KEG in pGADT7gate. 10x and 100x dilutions of transformed yeasts were spotted on selective medium lacking His (-3). Only JAZ12 interacts directly with the RING-domain mutant KEG(AA) in the LexA-based Y2H system. The EGY48 (p8opLacZ) yeast strain was co-transformed with KEG(AA)in pGILDA and JAZ in pB42AD. Transformed yeasts were spotted on inducing Gal/Raf medium supplemented with X-Gal. C, partial alignment of the amino acid sequences of JAZ12 and its orthologs from other plant species. D, alignment of the Jas motif of Arabidopsis JAZ proteins. C-D, Amino acids essential for COI1 interaction are marked with an asterisk, those mutated to test KEG interaction with a circle. Arabidopsis JAZ12 is boxed. E-F, Confocal root tip imaging of Arabidopsis seedlings stably overexpressing the JAZ12-GFP fusion protein. Propidium iodide staining was used to enhance visualization of the cells.. Scale bars are 30 µm (E) and 15µm (F).

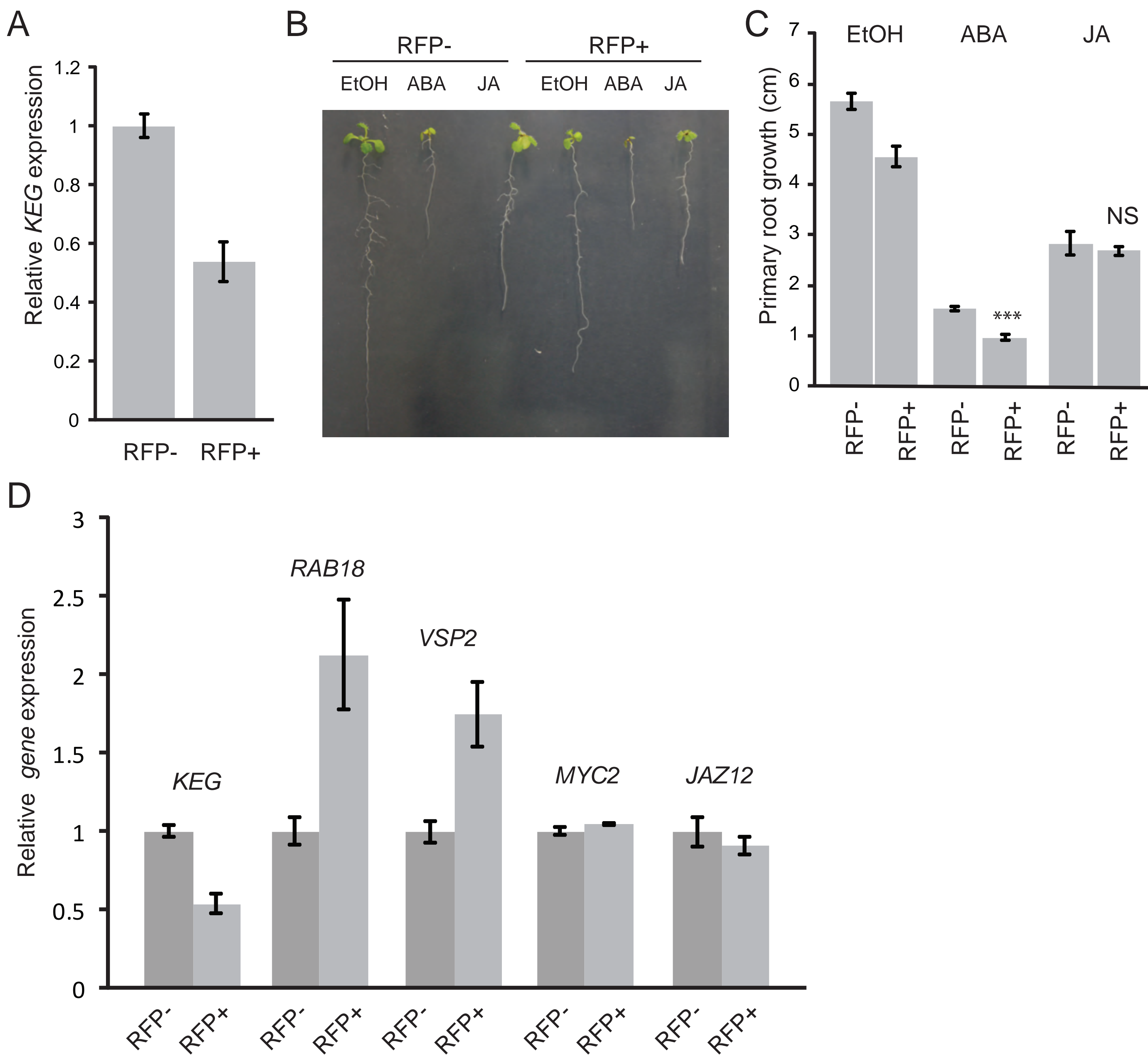
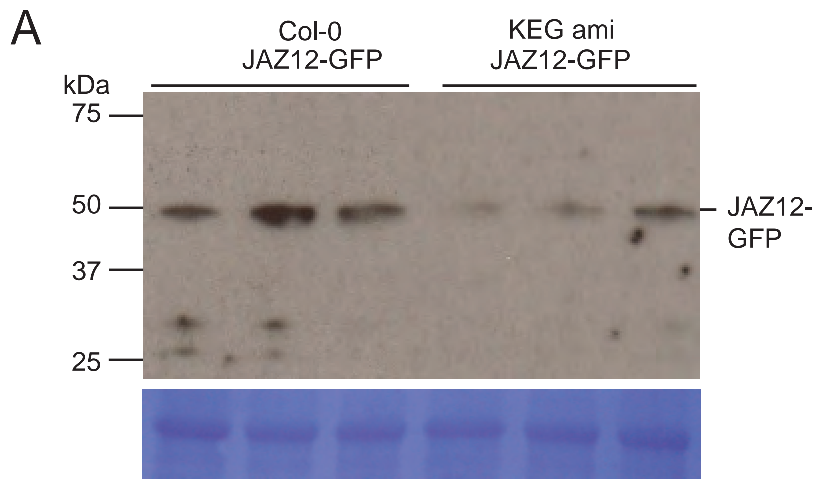


Figure S3. KEG knock down lines are hypersensitive to ABA. Seeds of KEG amiRNA Line 2 were separated based on RFP fluorescence using an epifluorescence microscope. Plants were grown for 4 days and transferred to media containing EtOH (control), 5 µM ABA or 2.5 µM JA for 6d. A, relative *KEG* expression. B, Representative 11-day-old WT and KEG knock down lines. C, quantification of increase in primary root length after transfer in (A). Error bars depict standard error (n=3). Treatment × genotype interaction effect (two-way ANOVA) is indicated (***, p-value < 0.001). There were also a significant effects for ABA treatment and of the genotype (P<0.001), and for JA treatment (p<0.05). D, expression of *KEG*, *RAB18*, *VSP2*, MYC2 and J*AZ12* in Line 2. Error bars depict standard error (n=3).



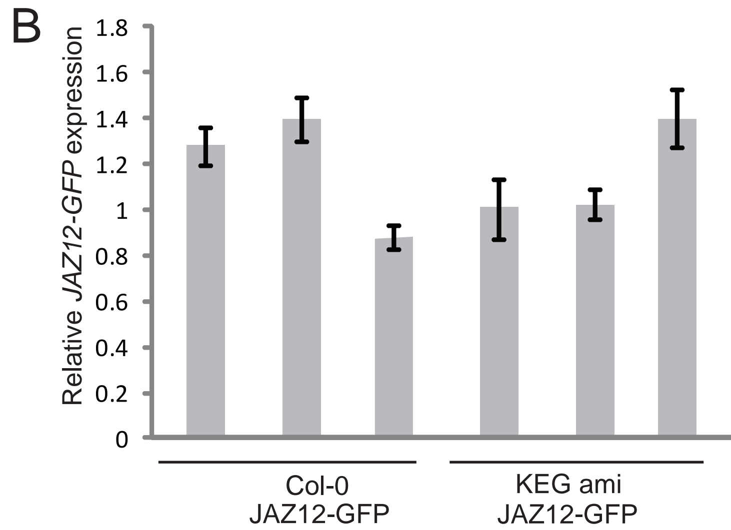


Figure S4. KEG is a positive regulator of JAZ12 stability. A, Immunoblot showing JAZ12-GFP levels in 3 biological repeats of a JAZ12-GFP line and its cross with the KEG amiRNA line 14. 6-day-old seedlings were used. Membrane was stained with Coomassie to inspect equal loading. B, *JAZ12-GFP* transcript levels in samples used in A. Error bars depict standard error of 3 technical replicates.

Table SI. Primers used in this study

ID		Sequence	Use
JAZ12 pair 1	Fw	CATCTAATGTGGCATCACCAG	qPCR
JAZ12 pair 1	Rv	TGCCTCCTTGCAATAGGTAGA	qPCR
JAZ12 pair 2	Fw	CTATCATGTACGCTGCTGTGTG	qPCR
JAZ12 pair 2	Rv	CCACTCCCAGACATGGAAAC	qPCR
JAZ12(ZIM)	Rv	AGAAAGCTGGGTTTTTAGCAGCAATACGAAGGA	Cloning
JAZ12(Jas)	Fw	AAAAAGCAGGCTACGCAATGGAGACAAAGAATTC	Cloning
JAZ12-1	Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTC**TCM**ATCAAAAACTGAATAAAATAG	Cloning
JAZ12(RR142/143AA)	Fw	CGGCTGATCTACCTATTGCA**GCGGCG**CATTCGCTTC	Mutagenesis
JAZ12(RR142/143AA)	Rv	TGCAATAGGTAGATCAGCCGTGGATCTGCA	Mutagenesis
JAZ12(F149A)	Fw	AGGCATTCGCTTCAACGAGCCCTCGAGAAAAGA	Mutagenesis
JAZ12(F149A)	Rv	TCGTTGAAGCGAATGCCTCCTTGCAATAGG	Mutagenesis
JAZ12(H144A)	Fw	CTACCTATTGCAAGGAGGGCTTCGCTTCAACG	Mutagenesis
JAZ12(H144A)	Rv	CCTCCTTGCAATAGGTAGATCAGCCGTGGA	Mutagenesis
JAZ12(P164A)	Fw	GTCAACAAAAACCCTTACGCTACTTCAGACT	Mutagenesis
JAZ12(P164A)	Rv	GTAAGGGTTTTTGTTGACCAATCTGTCCCG	Mutagenesis
JAZ12 (LVNKN157-161AAAAA)	Fw	GAGAAAGACGGGACAGAGCGGCTGCCGCAGCCCCTTACCCTACT	Mutagenesis
JAZ12 (LVNKN157-161VTSKA)	Fw	GAGAAAAGACGGGACAGAGTGACCTCCAAAGCCCCTTACCCTACT	Mutagenesis
JAZ12 (LVNKN157-161)	Rv	TCTGTCCCGTCTTTTCTCGAGGAATCGTTG	Mutagenesis
KEG amiRA	miR-s	GATTCTCTACTAACGTACAGCTATCTCTCTTTTGTATTCC	Cloning
KEG amiRA	miR-a	GATAGCTGTACGTTAGTAGAGAATCAAAGAGAATCAATGA	Cloning
KEG amiRA	miR*s	GATAACTGTACGTTACTAGAGATTCACAGGTCGTGATATG	Cloning
KEG amiRA	miR*a	GAATCTCTAGTAACGTACAGTTATCTACATATATATTCCT	Cloning
jaz12-1	Fw	AGTTATGGCACACTCCCATTG	Genotyping
jaz12-1	Rv	AGCATCAGTCCTGTCTCATCG	Genotyping

SALK LB1.3	-	ATTTTGCCGATTTCGGAAC	Genotyping
KEG	Fw	TTTGATGGACAGGTGCTTTG	qPCR
KEG	Rv	GAGCAACATCAGCCCCATA	qPCR
RAB18	Fw	GGCTTGGGAGGAATGCTT	qPCR
RAB18	Rv	TTGATCTTTTGTGTTATTCCCTTCT	qPCR
VSP2	Fw	ATGCCAAAGGACTTGCCCTA	qPCR
VSP2	Rv	CGGGTCGGTCTTCTCTGTTC	qPCR
MYC2	Fw	TCCGAGTCCGGTTCATTCT	qPCR
MYC2	Rv	TCTCGGGAGAAAGTGTTATTGAA	qPCR
GFP	Fw	GAAGCGCGATCACATGGT	qPCR
tNOS	Rv	ATTGCCAAATGTTTGAACGA	qPCR
UBC	Fw	CTGCGACTCAGGGAATCTTCTAA	qPCR
UBC	Rv	TTGTGCCATTGAATTGAACCC	qPCR
PP2A	Fw	TAACGTGGCCAAAATGATGC	qPCR
PP2A	Rv	GTTCTCCACAACCGCTTGGT	qPCR
JAZ10	Fw	AAAAAGCAGGCTCGATGTCGAAAGCTACCATAGA	qPCR
JAZ10	Rv	AGAAAGCTGGGTTTAGGCCGATGTCGGATAGT	qPCR
AFB2	Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAATTATTTCCCAGATG	Cloning
AFB2	Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCMGAGAATCCACACAAATGGCG	Cloning
COI1	Fw	AAAAAGCAGGCTATCCGATGGAGGATCCTGATA	Cloning
COI1	Rv	AGAAAGCTGGGTATATTGGCTCCTTCAGGACT	Cloning