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RESEARCH ARTICLE

The Arabidopsis Leucine-rich Repeat Receptor Kinase BIR3 Negatively Regulates BAK1 Receptor Complex Formation and Stabilizes BAK1

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Short title: BIR3 negatively regulates and stabilizes BAK1

One-sentence summary: The receptor kinase BIR3 negatively regulates cell surface receptor complexes and thereby prevents unwanted activation of immune and hormone responses.

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ABSTRACT

BAK1 is a co-receptor and positive regulator of multiple ligand-binding leucine-rich-repeat receptor kinases (LRR-RKs) and is involved in brassinosteroid (BR)-dependent growth and development, innate immunity and cell death control. The BAK1-interacting LRR-RKs BIR2 and BIR3 were previously identified by proteomics analyses of *in vivo* BAK1 complexes. Here we show that BAK1-related pathways such as innate immunity and cell death control are affected by BIR3 in *Arabidopsis*

thaliana. BIR3 also has a strong negative impact on BR signaling. BIR3 directly interacts with the BR receptor BRI1 and other ligand-binding receptors and negatively regulates BR signaling by competitive inhibition of BRI1. BIR3 is released from BAK1 and BRI1 after ligand exposure and directly affects the formation of BAK1 complexes with BRI1 or FLAGELLIN SENSING2. Double mutants of *bak1* and *bir3* show spontaneous cell death and constitutive activation of defense responses. BAK1 and its closest homolog BKK1 interact with and are stabilized by BIR3, suggesting that *bak1 bir3* double mutants mimic the spontaneous cell death phenotype observed in *bak1 bkk1* mutants via destabilization of BIR3 target proteins. Our results provide evidence for a negative regulatory mechanism for BAK1 receptor complexes in which BIR3 interacts with BAK1 and inhibits ligand-binding receptors to prevent BAK1 receptor complex formation.

INTRODUCTION

Plants recognize external signals using cell surface receptors to perceive their environment or developmental status and adapt to changing needs. In *Arabidopsis thaliana*, members of the receptor kinase family, one of the largest protein families, facilitate the perception of a wide spectrum of signals and the activation of downstream signaling (Lehti-Shiu et al., 2009a). Leucine-rich repeat receptor kinases (LRR-RK), the largest subfamily of receptor kinases, function in many aspects of plant growth, development and interaction with the environment (Gou et al., 2010). The BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED KINASE (BAK1) belongs to a five-member LRR-RK subfamily with five LRRs, called SOMATIC EMBRYOGENESIS RECEPTOR KINASE1 to 5 (SERK1-5) (Hecht et al., 2001). BAK1/SERK3 is a general regulator of other LRR-RKs (Chinchilla et al., 2009) by acting as an interactor and positive regulator of ligand-binding receptors (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011; Ladwig et al., 2015). The best-studied BAK1 interaction partners are FLAGELLIN SENSING2 (FLS2), which senses bacterial flagellin (or the derived epitope flg22), and BRI1, the major *Arabidopsis thaliana* brassinosteroid (BR) receptor. Biochemical and genetic analyses revealed that BAK1 is involved in both the BRI1 and FLS2 signaling pathways (Li et al., 2002; Nam and Li, 2002; Chinchilla et al., 2007; Heese et al., 2007). Analyses of the crystal structures of the ligand-bound trimolecular receptor complexes have shown how SERK co-receptors bind to ligand-binding LRR-RKs as well as to the receptor-bound ligands (Santiago et al., 2013; Sun et al., 2013a; Sun et al., 2013b). Ligand-induced association with co-receptors is essential for transmembrane activation of RKs (Song et al., 2016; Hohmann et al., 2017). Subsequent transphosphorylation steps lead to full activation of the cytoplasmic kinase domains and the initiation of signaling (Wang

et al., 2008; Cao et al., 2013; Bojar et al., 2014). Besides FLS2 and BRI1, BAK1 can interact with several other LRR-RKs such as the endogenous *At*PEP peptide RECEPTOR1 and 2 (PEPR1/PEPR2) (Postel et al., 2010; Tang et al., 2015), ELONGATION FACTOR TU RECEPTOR (EFR) (Roux et al., 2011), the PHYTOSULFOKINE RECEPTOR (PSKR1) (Ladwig et al., 2015; Wang et al., 2015a) and ERECTA (Meng et al., 2015). In addition, interaction of BAK1/SERK3 with the LRR-RK SUPPRESSOR OF BAK1-INTERACTING RECEPTOR1 (SOBIR1) as part of a bimolecular receptor-like protein (RLP) complex was reported (Gust and Felix, 2014; Albert et al., 2015; Meng et al., 2015; Postma et al., 2016), expanding the spectrum of BAK1- to RLP-mediated processes.

Reduced levels or overexpression of *BAK1* leads to deregulated cell death, indicating that a balanced receptor/co-receptor ratio needs to be maintained to prevent autoimmune cell death (He et al., 2007; Kemmerling et al., 2007; Dominguez-Ferreras et al., 2015). Double mutants of *bak1* with mutants of its closest homolog BAK1-LIKE1 (BKK1)/SERK4 strongly enhance the cell death phenotype of the *bak1* mutants, leading to seedling lethality in double mutant nulls (He et al., 2007). Mutant combinations with the weaker *bak1-3* allele show strong dwarfism and spontaneous cell death but no seedling lethality (Albrecht et al., 2008). BAK1 also interacts with a small LRR-RK called BAK1-INTERACTING RECEPTOR-LIKE KINASE1 (BIR1), which also has a strong effect on cell death control (Gao et al., 2009) and with its close relative BIR2 (Halter et al., 2014b). Both proteins belong to the BIR family of LRR-RKs subgroup Xa, with four members (BIR1 to BIR4). Loss-of-function mutants of *BIR2* have a similar effect on cell death control to that described for *BAK1*. Furthermore, BIR2 is a negative regulator of BAK1-mediated immunity. BIR2 acts by constitutively interacting with BAK1 in the absence of ligands and preventing unwanted interactions with ligand-binding receptors. After ligand activation, BIR2 is released from the complex and BAK1 can associate with the ligand-bound receptor complex partners. BIR2 affects flg22- and elf18 (bacterial elongation factor Tu peptide epitope)-induced signaling, as well as cell death control, but not BR signaling (Halter et al., 2014b). Other negative regulators of RKs have been identified, highlighting the importance of tight regulation of these cell surface perception complexes (for review see:(Couto and Zipfel, 2016)).

Here, we describe an Arabidopsis BIR family protein, BIR3, which has a strong impact on both BR- and microbe associated molecular pattern (MAMP)-induced

responses, but only weakly affects cell death control, showing that partially redundant but also distinct functions have evolved within the BIR family. Unlike the more specialized function of BIR2, BIR3 exhibits a general molecular mechanism through its additional interaction with and competitive inhibition of ligand-binding receptors.

RESULTS

BIR3 is a negative regulator of BR responses, MAMP and cell death signaling

BIR3 was first identified by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analyses of *in vivo* BAK1 complexes (Halter et al., 2014b; van Dongen et al., 2017). BIR3 is expressed in leaves and all other tissues of Arabidopsis (Supplemental Figure 1A, B) and is plasma membrane-localized (Supplemental Figure 1C), and it therefore resides in the correct cell compartment for potential interactions with other RKs such as BAK1. The interaction with BAK1 was confirmed by directed co-immunoprecipitation (Co-IP) (Supplemental Figure 1D, E), Förster resonance energy transfer-fluorescence lifetime imaging (FRET-FLIM), yeast-two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) (Halter et al., 2014b). BIR3 is predicted to encode an LRR-RK with five LRRs and a cytoplasmic kinase domain that lacks several conserved residues that are thought to be necessary for kinase activity (Supplemental Figure 2) (Halter et al., 2014b). We tested the kinase activity of BIR3 using *in vitro* kinase assays and could not detect any autophosphorylation activity of the BIR3 kinase domain (KD) or transphosphorylation of BAK1 (Supplemental Figure 3A). Weak transphosphorylation of BIR3 by BAK1 and BRI1 was detectable and was more pronounced with GST-tagged than with the His6-tagged recombinant fusion proteins (Supplemental Figure 3B). Thus, in contrast to BIR1 (Gao et al., 2009), but similar to BIR2 (Halter et al., 2014b), BIR3 most likely functions without the need for an enzymatically active kinase domain. To further analyze this BAK1-interacting protein, we generated overexpression lines (Supplemental Figure 4, 5). Overexpression of *BIR3* led to a dwarf phenotype (Figure 1 A) that was gene dosage-dependent but independent of the tag used for fusion proteins (Supplemental Figure 5). In strong homozygous overexpression lines, the morphology of these plants resembled that of *bri1* null mutants (Clouse et al., 1996), with dark curly leaves and a rosette diameter of about 0.9 cm (Figure 1 A, B, Supplemental Figure 5). Indeed, roots and hypocotyls of *BIR3*-

FLAG overexpressing plants were insensitive to exogenously applied brassinolide (BL) over a wide range of concentrations (Figure 1 C, Supplemental Figure 6A-D).

The positive regulatory transcription factor BRI1-EMS-SUPPRESSOR1 (BES1) is dephosphorylated in response to BL and relocates to the nucleus to activate BL responsive genes (Yin et al., 2002). This effect remained undetectable in *BIR3-FLAG* overexpressing plants, as BES1 primarily exists in the phosphorylated state (Figure 1 D). In wild-type Arabidopsis, multiple genes are down-regulated in response to BR treatment *via* a negative feedback mechanism (Mathur et al., 1998). We therefore analyzed the transcript levels of the BR-responsive genes *CONSTITUTIVE PHOTOMORPHOGENIC DWARF (CPD)* and *DWARF4* (*DWF4)* using qRT-PCR. The expression levels of both genes were significantly increased in *BIR3-FLAG* overexpressing seedlings compared to wild type Col-0 seedlings, indicating that BL signaling is reduced in these lines. After treatment with 1 µM 24-epiBL for 1 h, the relative expression levels of *DWF4* and *CPD* in *BIR3-FLAG* overexpressing plants were less reduced than those in wild-type (wt) Col-0 seedlings (Figure 1 E, F). Together, these data show that the dwarf phenotype of *BIR3* overexpressing plants is caused by (almost) complete insensitivity to BL (Figure 1 C-F, Supplemental Figure 6).

To determine if BIR3 also plays a role in other BAK1-dependent signaling pathways, we tested immune responses such as flg22- or elf18-induced reactive oxygen species (ROS) production and *FLAGELLIN-INDUCED RECEPTOR-LIKE KINASE1 (FRK1)* gene expression, bacterial growth, as well as cell death induced by the necrotrophic fungus *Alternaria (A.) brassicicola*. The MAMP-induced ROS burst was strongly reduced in hemizygous *BIR3* overexpressing plants compared to wild type, as was seedling growth inhibition by flg22 (Figure 2 A, Supplemental Figure 7). Flg22-induced *FRK1* marker gene expression was also reduced in these lines (Figure 2 B), confirming that BIR3 is also a negative regulator of flg22 responses. After infection of *BIR3* overexpressing plants with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000), no differences in bacterial growth were detectable (Figure 2 C). After infection with the necrotrophic fungus *A. brassicicola,* cell death responses were stronger than those in wt plants, indicating that BIR3 either negatively regulates defense responses against this non-pathogenic fungus or is involved in cell death control (Figure 2 D, E). This phenotype resembles that of *bak1* mutants, which are impaired in MAMP responses and show stronger cell death

reactions than wild type (Kemmerling et al., 2007). These antagonistic effects result in no alterations in bacterial growth (Roux et al., 2011). Taken together, these results indicate that BIR3 negatively affects BR and MAMP responses as well as cell death control.

BIR3 interacts with different ligand-binding receptors and competitively inhibits BRI1 signaling independently of BAK1

Because of the strong BR phenotype of *BIR3* overexpressing plants, we tested for a protein–protein interaction between BIR3 and the BR receptor BRI1. Co-IP after transient expression of BRI1-HA and BIR3-YFP in *Nicotiana benthamiana* showed that BIR3 indeed associates with BRI1 (Figure 3 A). Endogenous BRI1 also bound to BIR3-GFP in stably transformed Arabidopsis plants (Figure 3 B). Further evidence for a BIR3-BRI1 interaction came from LC/MS/MS analyses of BRI1-FLAG immunoprecipitates, which identified at least five unique high-scoring BIR3 peptides (Supplemental Table 1, Supplemental Figure 8), thus providing additional support for the association of BIR3 and BRI1 *in vivo*. Furthermore, expression of BRI1-GFP in the *BIR3-FLAG* overexpressing background led to the complete complementation of the dwarf phenotype normally observed upon *BIR3* overexpression (Figure 3 C, Supplemental Figure 9). These results show that BIR3 competitively inhibits BR signaling. That the *BIR3* overexpression phenotype can be compensated by enhancing BRI1 levels points to a direct effect of BIR3 on the BRI1 receptor complex.

BIR3 can also interact with FLS2, EFR and PEPR1, as shown by Co-IP after transient expression in *N. benthamiana* (Figure 3 D, E). In addition to its interaction with BAK1, BIR3 interacts with all ligand-binding LRR-RKs tested. To test whether these interactions are direct or BAK1-mediated, we performed yeast split ubiquitin system (SUS) assays, which revealed a likely direct interaction of full-length BAK1 and BIR3 (Figure 4 A). Although weaker than the BAK1-BIR3 interactions, BAK1-BRI1 and BRI1-BIR3 interactions, as well as BIR3-FLS2 interactions, were observed in yeast (Figure 4 A, Supplemental Figure 10). The direct interaction of BIR3 with BRI1 was also confirmed by BiFC assays showing reconstitution of YFP when the N- and C-terminal part of YFP were fused to BIR3 and BRI1, respectively, and transiently expressed in *N. benthamiana* (Figure 4 B), suggesting that both proteins exist in very close proximity *in planta*.

We also investigated if BIR3, BAK1 and BRI1 form a tripartite complex. We performed split-ubiquitin bridge (SUB) assays (Grefen, 2014) to test whether BIR3 or BAK1 influences the interaction of the respective two other proteins. BIR3 did not enhance the interaction of BAK1 and BRI1, nor did BAK1 enhance the interaction of BIR3 and BRI1 (Figure 4 C, D; Supplemental Figure 10), pointing to an independent and direct interaction of BIR3 and BRI1 that is not affected or mediated by BAK1. Interaction of BIR3 and BRI1 was also detected in the absence of BAK1 in plants overexpressing *BIR3-FLAG* in the *bak1-4* mutant background (Figure 4 E). Together, these data explain the strong phenotype of the *BIR3* overexpression lines and the competitive inhibition of BRI1 by BIR3 via an additional direct and BAK1-independent interaction and inhibition of the ligand-binding receptor.

BIR3 is released from BAK1 complexes after ligand binding to their receptors

BIR2 is released from BAK1 after ligand binding to the respective receptors (Halter et al., 2014b). We therefore tested if this was also the case for BIR3. BIR3 was also partially released from BAK1 after flg22 and BL treatment *in planta* (Figure 5 A, B). The same phenomenon was observed after *At*Pep1 treatment (Figure 5 A). Only a fraction of BAK1 was set free after a single ligand treatment, but treatment with a mixture of flg22, elf18, BL and *At*Pep1 led to a significantly higher amount of BAK1 being released from the complexes with BIR3 (Figure 5 A), supporting the finding that BAK1 exists in receptor-specific subpools that can only be addressed by the respective ligand (Halter et al., 2014a).

BIR3 negatively regulates complex formation of BAK1 with ligand-binding receptors

BIR3 is a negative regulator of BR and MAMP responses and constitutively interacts with BAK1 in the absence of ligands. To determine how BIR3 regulates different pathways, potentially by directly affecting receptor complex formation at the plasma membrane, we tested complex formation of BAK1 and FLS2 in *bir3* mutants and *BIR3* overexpressing plants. The amount of FLS2 that associated with BAK1 after flg22 treatment was significantly higher in the *bir3* mutants than in wild type. In *BIR3* overexpressing lines, an FLS2-BAK1 interaction was not detectable (Figure 5 C), showing the very strong and direct impact of BIR3 on FLS2-BAK1 complex formation. Testing for association of BRI1 and BAK1 in the absence and presence of BIR3

revealed the same effect, with less BAK1 being associated with BRI1 when BIR3-GFP was stably expressed in Arabidopsis (Figure 5 D). Treatment with BL was not necessary for this interaction, as BAK1 and BRI1 already interacted in the absence of exogenous ligand application, likely due to the presence of endogenous BR. Therefore, BIR3 executes its negative regulatory function with the same molecular mechanism as described for BIR2: by a direct inhibitory effect on FLS2-BAK1 and BRI1-BAK1 complex formation.

***bir3* mutant lines show unexpectedly weak phenotypes**

The above described effects of *BIR3* overexpression on MAMP and BR responses, and on the corresponding receptor complexes, would suggest that opposite phenotypes should be detectable in *bir3* mutants. This is indeed the case for MAMP responses, as shown with two independent *bir3* alleles. Neither allele expressed full-length *BIR3* transcripts nor detectable amounts of protein, so both are considered null mutants (Supplemental Figure 11). These mutants showed opposite phenotypes compared to the *BIR3* overexpressing lines, with stronger seedling growth inhibition and enhanced ROS bursts after flg22 or elf18 treatment (Figure 6 A, B, Supplemental Figure 12). However, BL responses such as hypocotyl growth inhibition, root growth inhibition, BES1 dephosphorylation and *CPD* or *DFW4* expression were not significantly altered in the *bir3* mutants compared to wild type (Figure 6 C, D; Supplemental Figure 6, 13), although we detected weak, positive effects of up to 100 nM BL treatment on hypocotyl growth in the mutants, and BES dephosphorylation was slightly more complete in the mutants than in Col-0 plants. In double mutants with the weak *bri1-301* allele (Xu et al., 2008), even a negative influence of the loss of *BIR3* became apparent during this long-term experiment (Figure 6 E-G). This result indicates that BL and MAMP responses are differentially sensitive to the loss of *BIR3*. Also, susceptibility to *Pto* DC3000 and *A. brassicicola* was not altered in the mutants (Supplemental Figure 14). The weakness of the MAMP and BL phenotypes cannot be explained by the previously described function of BIR3. However, another phenotype of the *bir3* mutants became evident that explains the weak phenotypes of the *bir3* mutants in contrast to the strong effects of *BIR3* overexpression.

BIR3 stabilizes BAK1 and other SERK proteins

Single *bir3* mutants are not impaired in cell death control (Supplemental Figure 14). In combination with *bak1-4* and the weaker *bak1-3* allele (Albrecht et al., 2008), *bak1 bir3* double mutant plants are small, have curly leaves and show spontaneous cell death (Figure 7 A,B). Salicylate (SA) and jasmonate (JA) levels were significantly enhanced and, as a consequence, SA and JA-responsive marker gene expression (*PR1* and *PDF1.2*, respectively) was elevated in these plants compared to wild type (Figure 7 C-F). These results indicate that the dwarf phenotype of the *bak1 bir3* double mutants is likely caused by constitutive activation of defense responses and cell death. We also used these double mutants for complementation analyses, as the single mutants showed only weak phenotypes. Both BAK1 and BIR3 expressed in the double mutant background restored the dwarf phenotype (Supplemental Figure 15), showing that these morphological changes are indeed caused by the lack of BAK1 and BIR3. Protein gel blot analysis of endogenous BAK1 in the *bir3* mutant background showed that BAK1 levels were significantly reduced in the *bir3* mutants compared to wild type (Figure 5C, 8 A). In addition, BKK1 levels were enhanced when this protein was transiently expressed together with BIR3 in *N. benthamiana* (Figure 8 B), indicating that BIR3 stabilizes SERK family proteins. The cytoplasmic domains of BIR3 and SERKs also interacted in yeast two-hybrid assays and *in vitro* pulldown assays (Supplemental Figure 16), and BIR3 associated with BKK1 when transiently expressed in *N. benthamiana* (Figure 8 C). The *bak1 bir3* double mutants resembled the phenotype of *bak1 bkk1* double mutants when the weak *bak1-3* allele was used (Albrecht et al., 2008) (Figure 8 D). The very similar phenotypes of *bak1 bir3* and *bak1 bkk1* double mutants, along with the stabilizing activity of BIR3 and the potential to interact with BAK1 and BKK1, provides a potential explanation for the spontaneous cell death phenotype as a consequence of SERK protein destabilization in the absence of BIR3.

This explanation could account for the observation that the single mutant phenotypes were so weak. The destabilization of BAK1 antagonizes the negative regulatory action of BIR3 that would result in stronger responses in the mutants. However, as the positive regulator BAK1 is destabilized in the mutants, this negative effect is leveled out and results in weak if any phenotypes. To test this hypothesis, we expressed BAK1 under its native promoter in the *bak1-4 bir3-2* double mutant background and compared this line to the *bir3-2* mutants, finding that the BAK1-expressing lines showed stronger BL responses (Figure 9 A-D). In addition, the

typically elongated petioles and leaves of these lines are indicative of enhanced BL responsiveness (Supplemental Figure 15). One could argue that this effect is due to increased expression of the positive regulator BAK1. To test this, we created isogenic lines expressing BAK1 in the *bak1-4* single mutant background as a control. We measured BAK1 expression in both lines and found that BAK1-GFP levels were slightly lower in the double mutant background compared to the control (Figure 9 E). Again, the responses were stronger in the BAK1-GFP-expressing double mutants, even compared to the isogenic BAK1-GFP *bak1-4* control line, which expressed slightly more BAK1 (Figure 9 E-I). This experiment clearly showed that the negative regulatory function of BIR3 becomes evident when the destabilizing effect of BIR3 is partially overcome by ectopic expression of BAK1.

DISCUSSION

BAK1 is well-known as a general regulator of ligand-binding RKs (Chinchilla et al., 2009; Liebrand et al., 2014). Several of these interactions have been studied; however, few generalizable rules have emerged about BAK1-RK interactions thus far. BAK1 can interact with BRI1 in the absence of (exogenous) ligands. The release of the BRI1 KINASE INHIBITOR1 (BKI1) upon BL activation of BRI1 allows for the efficient association of BAK1 and BRI1, and sequential transphosphorylation events lead to the activation of BR responses (Li et al., 2002; Nam and Li, 2002; Russinova et al., 2004; Wang et al., 2008; Jaillais et al., 2011). While BRI1 and BAK1 are highly active RD-kinases, FLS2 and EFR are non-RD kinases (Dardick and Ronald, 2006). They do not interact with BAK1 in the absence of ligands (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011), and kinase activity is only activated after ligand binding. Moreover, the activation of BAK1 leads to phosphorylation of BOTRYTIS-INDUCED KINASE1 (BIK1), which in turn phosphorylates BAK1 and FLS2 and activates downstream responses such as ROS production (Lu et al., 2010; Zhang et al., 2010). BIR1 interacts constitutively with BAK1 but not with other RKs such as FLS2, PEPR1 and CLAVATA and plays a role in cell death control but not in BR or MAMP signaling (Gao et al., 2009; Liu et al., 2016). BIR2 also constitutively interacts with BAK1 and is released after ligand binding to BAK1-interacting RKs, allowing for the interaction of BAK1 with the ligand-bound RKs. BIR2 negatively influences BAK1-RK complex formation via a direct interaction with BAK1, but not with ligand-binding receptors, and it plays a role in cell death control and MAMP

signaling but not in BR responses (Halter et al., 2014b). Together with BIR2, BIR3 was found to comprise part of *in vivo* BAK1 complexes. Arabidopsis plants overexpressing *BIR3* showed a very strong dwarf phenotype characterized by almost complete BL insensitivity. Impairment in BR signaling is a phenotype that was not observed for *bir1* or *bir2*, while impairment in cell death control, which is a major trait of *bir1* and, to a lesser extent, of *bir2* mutants, was absent in *bir3* single mutants. These results indicate that BIR proteins have overlapping and distinct functions that evolved after the duplication of these *RK* genes.

In Co-IP experiments, BIR3 was found in BRI1 complexes, suggesting that BIR3 might use an alternative mechanism to exert a strong impact on BR signaling. From these experiments, it was not clear whether this is a direct interaction of BRI1 and BIR3 or an indirect interaction mediated by BAK1. Split-ubiquitin assays revealed a direct interaction of BIR3 with BRI1, which was confirmed in BiFC assays *in planta*. It is unlikely that a tripartite complex forms, as in split-ubiquitin bridge assays, none of the tested combinations with BAK1 or BIR3 with the two other proteins showed enhanced interactions. The observation that BIR3 directly interacts with BRI1 in a BAK1-independent manner suggests that BIR3 interacts with and negatively regulates both BAK1 and BRI1 independently. Therefore, BIR3 uses a mechanism distinct from that of BIR1 and BIR2 to exert a strong negative effect on BR signaling. BIR3 can interact with other ligand-binding receptors such as FLS2, EFR, and PEPR1, providing additional evidence that BIR3 not only interacts with BAK1, as shown for BIR1 and BIR2, but it also interacts with ligand-binding receptors to negatively regulate complex formation and downstream signaling.

This notion is supported by the finding that *BRI1* overexpression rescued the *BIR3* overexpression phenotype, pointing to a competitive inhibition of BRI1 by BIR3 that can be overcome by enhancing BRI1 levels. The quantitative effects of hemizygous BIR3 overexpression also support the model of competitive inhibition, since higher *BIR3* expression levels led to stronger blocking of the BR responses. Homozygous *BIR3* overexpressing lines were largely insensitive to BL and resembled *bri1* null mutants. BIR3 can interact with all functional SERK proteins (Supplemental Figure 16). If BIR3 solely blocks BAK1 and other SERKs, *BRI1* overexpression would not be able to rescue the BR phenotype, as SERKs are indispensable for BR signaling (Gou et al., 2012). Complementation of *BIR3* overexpressing lines by *BRI1* overexpression was complete, even when BIR3 was

expressed at high levels. Complementation with *BAK1* overexpression was only partial, showing that BRI1 complementation is more effective (Supplemental Figure 17). In the absence of BAK1, *BIR3* overexpression still led to a dwarf phenotype, showing that proteins other than BAK1 must be targets of BIR3 (Supplemental Figure 18). This demonstrates that BIR3 uses two independent mechanisms to block BR responses: it interacts with BAK1 and other SERKs, but also with BRI1, leading to a very efficient inhibition of BR responses that is not observed for BIR2.

BIR3 not only negatively regulates BR responses, but it also has a strong negative influence on MAMP signaling that coincides with the almost complete inhibition of FLS2-BAK1 complex formation. This blocks flg22-induced responses through a direct inhibitory effect on receptor complexes at the plasma membrane. As with BIR2, BIR3 was released from BAK1 after ligand-induced activation of the receptor complexes and, as shown for BIR2, this effect was dependent on the individual ligands used. Each ligand caused partial release of BIR3 from BAK1, but a mixture of different known ligands enhanced the release, supporting the existence of preformed complexes of BAK1 with individual ligand-binding receptors that can only be affected by their respective ligands (Bücherl et al., 2013). BIR3 could be considered to act as a ’bodyguard’ for BAK1 to keep its function under control in the absence of ligands. Like BIR2, BIR3 is kinase-inactive and likely functions as an inhibitor of BAK1 complex formation by interacting with BAK1 independently of any kinase activity. In contrast to BIR2, transphosphorylation of BIR3 is very weak and was only detectable in *in vitro* transphosphorylation assays when using a highly active GST-fusion construct, suggesting that the BAK1-BIR3 interaction may not be dependent on phosphorylation events and that BIR3 exerts its function only through its affinity to RKs (Supplemental Figure 3).

Cell death regulation is a common role of BIR1 and BIR2. In *bir3* single mutants, we were unable to detect elevated cell death, indicating that this trait has been gradually lost within the paralogous protein family. In combination with *bak1*, double mutants showed a severe dwarf phenotype with spontaneous cell death. The finding that BIR3 can interact with all SERKs and stabilizes them led us to the conclusion that the absence of BIR3 might destabilize BAK1 and BKK1, leading to a *de facto bak1 bkk1* mutant phenotype. The very similar phenotype of both double mutants confirms this observation, explaining the *bak1 bir3* double mutant phenotype as a cell death response caused by the absence of BAK1 and BKK1 (He et al.,

2007). This negative regulation is likely posttranscriptional, as *BAK1* and *BKK1* transcript levels were not altered in the *bir3* mutant compared to wild type (Supplemental Figure 19 A, B). Yamada et al. (2016) reported that depletion of BAK1 primes PEPR-mediated cell death responses, and de Oliveira et al. (2016) suggested that cysteine-rich receptor kinases (CRK) might be client proteins of protein glycosylation involved in BAK1-regulated cell death. Therefore, sensitizing pro-death RKs by depleting BAK1 via the loss of BIR3 might be involved in *bir3 bak1*-mediated cell death.

The *bir3* single mutants have only very weak phenotypes. MAMP responses are enhanced in these mutants, indicating that BIR3 negatively regulates MAMP responses, but the effects are rather weak and are absent for BR responses. The stabilization and sequestration of BAK1 might be two distinct functions of BIR3. The destabilizing activity of *bir3* antagonizes its effects on MAMP and BR responses. By destabilizing BAK1, the hyper-responsiveness of the *bir3* mutants might be masked by the negative effect of reduced BAK1 levels. Expression of BAK1-GFP in the *bak1 bir3* background led to a BL hyper-responsive growth phenotype (Supplemental Figure 15), with elongated leaves. This morphology resembles elongated mutants that are hyper-responsive to BL (Chung et al., 2012), confirming that the hyper-responsiveness phenotype of the *bir3* mutants becomes more evident when BAK1 levels are enhanced by ectopic expression. Moreover, these lines showed enhanced root growth inhibition after BL treatment and were less responsive to brassinazole (BRZ), an inhibitor of BL biosynthesis, treatment than BAK1 expressing plants in the *bak1-4* single mutant background (Figure 9), confirming the negative regulatory function of BIR3 on the BR pathway when BAK1 levels are stabilized by ectopic expression. This also confirms that expressing each of the two proteins in the *bak1 bir3* double mutant can rescue its dwarf phenotype, showing that indeed the loss of both BAK1 and BIR3 is the cause of this phenotype. This experiment also proves the functionality of BIR3-GFP fusion proteins.

Excessive activation of immune receptors can lead to deleterious consequences in mammals and plants, such as septic shock or autoimmune cell death (Singer et al., 2016; van Wersch et al., 2016). Therefore, the tight regulation and fine-tuning of receptor activation is essential. There are many examples showing that receptors need to be well controlled. In mammals, for example, soluble versions of TOLL-like receptors regulate receptor activation, likely by competing for ligand

binding with the full receptors and blocking signal transduction (Henrick et al., 2016). Other mechanisms include dissociation of adapter proteins, ubiquitination or de-ubiquitination, deactivation of the receptor complexes or interaction with inactive downstream partners such as the pseudokinase INTERLEUKIN RECEPTOR ASSOCIATED KINASE M (IRAK-M) (Kobayashi et al., 2002; Kawai and Akira, 2010). BAK1 is a central co-receptor of several LRR-RKs and RLPs and is therefore an ideal target for the regulation of multiple signaling pathways. Protein phosphatases (PP) such as PP2A (Segonzac et al., 2014) and KINASE ASSOCIATED PROTEIN PHOSPHATASE (KAPP) (Ding et al., 2007) negatively regulate BAK1 downstream signaling, likely by dephosphorylating BAK1 or the interacting RKs, respectively.

The pseudokinase BIR2 constitutively interacts with BAK1 and prevents BAK1 complex formation with ligand-binding receptors such as FLS2 (Halter et al., 2014b). BRI1, another LRR-RK with strong auto- and transphosphorylation activity, is also negatively regulated by a combination of multiple mechanisms: phosphorylation events, intramolecular inhibition by its own C-terminus and inhibition by interacting proteins such as BKI1, PP2A and BIK1 (Wang et al., 2005a; Wang and Chory, 2006; Wang et al., 2008; Jaillais et al., 2011; Oh et al., 2011; Wu et al., 2011; Oh et al., 2012; Lin et al., 2013; Wang et al., 2015b). These examples show that negative regulation at multiple levels and through multiple mechanisms is very important to assure balanced and fine-tuned activation and deactivation of RK signaling. BIR3 is a potent inhibitor of RK signaling by directly interacting with BAK1 and with ligand-binding receptors to negatively regulate receptor complex association, adding another component of negative receptor regulation to the network.

Although the BIR family proteins are very similar in terms of sequence (41-74% identical at amino acid residue level) and domain structure, our data suggest that plants have evolved new molecular functions and mechanisms after duplication events within the BIR subfamily of LRR-RKs. Here, we describe a mechanism for the negative regulation of RK-mediated processes. In contrast to the other BIRs, BIR3 associates not only with BAK1 but also with ligand-binding receptors such as FLS2 and BRI1. Direct interaction with the corresponding ligand-binding receptors results in a stronger inhibitory effect of BIR3 compared to BIR2 (Halter et al., 2014b). The affinity of BIR3 for BAK1 is used to stabilize BAK1, which does not accumulate to normal levels in the absence of BIR3. The stabilization of a positive regulator antagonizes the negative regulatory function of BIR3 and results in unexpectedly

weak or even negative effects in the mutants (Figure 10). It will be interesting to see whether these two functions can be dissected or if stabilization and negative regulation are both consequences of the affinity of BAK1 for BIR3. During evolution, new molecular functions and mechanisms have evolved after the duplication of *RK* genes (Lehti-Shiu et al., 2009b). The evolution of the different functions of the BIR family proteins, namely their influence on cell death control versus their inhibitory effects on LRR-RK-mediated signaling, will be an interesting topic for future analyses.

METHODS

Plant material and growth conditions

The *Arabidopsis thaliana* T-DNA insertion mutants used in this study are *bir3-1* (Salk_132078) and *bir3-2* (Salk_116632). Stable transgenic *Pro35S:BIR3* plants were obtained by floral dipping of pB2GW7-BIR3 into Col-0 wildtype plants (Clough and Bent, 1998). Stable transgenic lines containing *ProBAK1:BAK1-GFP* and *ProBIR3:BIR3-GFP* in the *bak1-4 bir3-2* background were constructed by *Agrobacterium*-mediated transformation of heterozygous *bak1-4-/- bir3-2+/-* and *bak1-4+/- bir3-2-/-* lines, respectively. Kanamycin-resistant seedlings were grown for three generations to obtain stable BAK1-GFP and BIR3-GFP transformants in a double-null *bak1-4 bir3-2* background, as well as isogenic BAK1-GFP transformants in a *bak1-4* background and BIR3-GFP transformants in a *bir3-2* background. Transformants were selected by genotyping for wt, T-DNA inserted and GFP-tagged *BAK1* and *BIR3* genes.

Plants were grown for 5 to 6 weeks on soil in growth chambers under short day conditions (8 hr light, 16 hr dark; 22°C; 110 μEm^{-2} s^{-1}, Osram lumilux cool white fluorescence tube lamps), for four weeks under long day conditions (16hr light, 8 hr dark, Osram lumilux cool white fluorescence tube lamps), or on ½ MS medium.

Constructs used in this work

Full-length and kinase domain constructs of BAK1 and BIR3 were obtained as described by (Kemmerling et al., 2007; Halter et al., 2014b). Full-length clones for the RLKs PEPR1, FLS2 and EFR were obtained from ABRC (provided by Steve Clouse) as N1g73080_ZEF and N5g646330_ZEF and N5G20480_ZEF. The *BRI1* coding sequence was amplified from cDNA with primers listed in Supplemental Table 2 and

cloned into pCR8 TOPO vector (Life Technologies). The *BKK1* coding sequence was amplified from cDNA with primers listed in Supplemental Table 2 and cloned into pCR8 TOPO vector (Life Technologies).

To create stable BIR3-overexpressing Arabidopsis plants, the *BIR3* coding sequence was cloned into pB2GW7 (35S promotor, no tag) (Karimi et al., 2002), and in pBIB-HYG (Gou et al., 2010) for C-terminal FLAG fusion, transformed into Agrobacterium strain GV3101, and used for floral dipping of Arabidopsis plants (Clough and Bent, 1998). The full-length genomic construct of BAK1 fused to GFP was obtained as described by (Albrecht et al., 2012). The endogenous promotor pBIR3-BIR3-GFP construct was generated by recombination of the following three entry clones and pK7m34GW: 1156 bp upstream promoter region of *BIR3* cloned with XhoI-SacII in pDONRP4-P1r; the *BIR3* gene amplified from genomic DNA and cloned into pENTRD-TOPO (Invitrogen) and pDONRP2r-P3-GFP (Karimi et al., 2007).

For transient protein expression in *N. benthamiana* under the control of the 35S-promotor, BIR3 full-length constructs were recombined into pB7YWG2 vector (Karimi et al., 2002) to obtain C-terminal YFP fusion and in pBIB-HYG for C-terminal FLAG fusion. *FLS2, PEPR1, EFR1* and *BKK1* were cloned into pGWB17 (Nakagawa et al., 2007) to obtain C-terminal 4xMYC fusions, *BRI1* and *BKK1* were cloned into pGWB14 (Nakagawa et al., 2007) to obtain C-terminal 3xHA fusions, and the *BRI1* coding sequence was cloned into pBIB-BASTA (Gou et al., 2010) for C-terminal GFP fusions.

For the yeast split-ubiquitin assays, the vectors pMetYC-Dest (Met repressible expression of protein with C-terminal Cub-ProteinA- LexA-VP16) and pXNubA22-Dest (constitutive expression of protein with C-terminal NubA-3xHA), pMetYC-BAK1 and pXNubA22-BRI1 were obtained from (Grefen et al., 2009). The full-length *BIR3* coding sequence was recombined into the Gateway vectors pXNubA22-Dest and pMetYC-Dest. For the split ubiquitin bridge assays, full-length *BIR3* and *BAK1* were recombined into pZMU-Dest.

For the yeast two hybrid assays, the cytoplasmic domains of *SERK1-4* were cloned into pGADT7 for activation domain fusions and the *BIR3* cytoplasmic domain was cloned into pGBKT7 for binding domain fusions. GST-BAK1 was described elsewhere (Wang et al., 2008).

For the BIFC assays, *BIR3* and *BRI1* were recombined into the Gateway vectors pUBC-cYFP and pUBC-nYFP (Grefen et al., 2010) to obtain C-terminal fusions to the N- or C-terminal half of YFP under the control of the *ubiquitin10* promotor.

For recombinant protein expression in *E. coli* and the kinase assays, the kinase domains of *BAK1, BAK1 K317E,* and *BIR3* were recombined into pDest15 to create GST-fusions and into pDest17 to create HIS6-fusions. The GST-BAK1 and FLAG-BRI1 constructs used in the BIR3 transphosphorylation assays are described elsewhere (Wang et al., 2008).

For the *in vitro* interaction assays, the cytoplasmic domains of *SERK1-4* were cloned into pFLAG-MAC for the FLAG fusions, and the cytoplasmic domains of *BIR1-3* were cloned into pMal-C2 for the MBP fusions.

All primers used are listed in Supplemental Table 2.

LC/MS/MS analysis

The BRI1-FLAG transgenic lines, plant growth conditions and IP protocols were as previously described (Wang et al., 2005b). LC/MS/MS analysis was performed on an Agilent 1100 series capillary LC system (Agilent Technologies, Palo Alto, CA) coupled directly online with an LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) using previously described conditions (Wang et al., 2005), except that MS/MS spectra were searched against the TAIR10 database with Mascot version 2.5.1.

Infection procedures

Pseudomonas syringae pv*. tomato* DC3000 infections were performed as described by Mosher et al. (2012). *Alternaria brassicicola* infection assays were carried out as described by Kemmerling et al. (2007).

Histochemical assays

Cell death and fungal mycelium was detected with trypan blue staining as described in Kemmerling et al. (2007).

Oxidative burst measurements

Oxidative bursts were measured using a luminol-based assay as described in Halter et al. (2014b).

Hormone measurements

Salicylate and jasmonate contents were measured as described by Lenz et al. (2011).

Kinase activity assays

Recombinant protein expression was performed as described in Halter et al. (2014b). Kinase activity assays were performed primarily as described by Schwessinger et al. (2011) but incubated for 1 hour at 37°C with shaking. Kinase assays using GST-BAK1 were performed as described by Wang et al. (2008).

RT-PCR analysis

Transcript levels were analyzed by standard or quantitative RT-PCR (qRT-PCR) using SYBR-green as a dye as described by Mosher et al. (2012) with primers listed in Supplemental Table 2.

In vitro interaction assays

All constructs were transformed into *E. coil* BL21(DE3) pLysS. SERK1, SERK2, BAK1 and BKK1 were purified with Anti-FLAG M2 Affinity Gel beads (Sigma-Aldrich) according to the manufacturer's protocols. MBP, MBP-BIR1, MBP-BIR2, MBP-BIR3 were immobilized with amylose resin (New England Biolabs) following standard protocols. Three micrograms of FLAG fusion proteins were preincubated with 10 µL prewashed amylose resin in 120 µL incubation buffer (1 mM NaCl, 20 mM $MgCl_2$, 0.2% Triton X-100, and 0.1 M HEPES at pH7.2) for 1 h at 4^{o}C. After centrifugation, the supernatant was collected and incubated with prewashed amylose resin with immobilized MBP or MBP fusion proteins at 4^{o}C for an additional 1 h. The resin was collected and washed 5 times with washing buffer. The pulled-down proteins were detected by protein blot with an anti-FLAG antibody.

Transient expression in *Nicotiana benthamiana*

Transient Expression in *Nicotiana benthamiana* was performed as described in Halter et al. (2014b).

Co-immunoprecipitations

Leaves were ground in liquid nitrogen, and 250 µl extraction buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P40, proteinase inhibitor cocktail (Roche)) was added to each 200 mg powdered tissue sample. The samples were homogenized and incubated for 1 h at 4°C under gentle shaking. The samples were centrifuged twice at 4°C and 14,000 rpm for 10 min to obtain a clear protein extract. After washing with extraction buffer, either 15 µl protein A agarose beads (Roche) were incubated for 1 h with 5 µl anti-BAK1 or anti-BIR3 antibody, or GFP-trap beads (Chromotec) were used. Supernatants containing equal amounts of protein were incubated for 1 h at 4°C with the beads. The beads were washed twice with 50 mM Tris-HCl pH 8.0, 150 mM NaCl and once with 50 mM Tris-HCl pH 8.0, 50 mM NaCl before adding SDS sample buffer and heating at 95°C for 5 min.

SDS-PAGE and immunoblotting

Proteins were separated, blotted, and incubated with antibodies as described by Schulze et al. (2010) but using 8% SDS gels and the following antibody dilutions: anti-GFP (Acris), 1:5,000; anti-c-myc (Sigma), 1:5,000; anti-HA (Sigma), 1:2,000; anti-BAK1 (Agrisera), 1:3,000; anti-FLS2, 1:2,500; anti-BRI1 (Agrisera) 1:5,000; anti-BIR3 antibodies were obtained from rabbits immunized with the peptide CVGSRDSNDSSFNN fused to KLH (Agrisera) 1:500; anti-BES1 1:2000 and anti-FLAG 1:2000 and anti-rabbit (Sigma), 1:50,000; anti-goat (Sigma), 1:10,000; anti-mouse (Sigma), 1:10,000. Chemiluminescence was detected with the ECL Western blotting detection system (GE Healthcare) and Kodak XJ300 film or a CCD camera (Amersham Imager 600). Quantification was done with ImageJ and ImageQuant software. Intensities of unsaturated bands were quantified and presented as relative values compared to the respective control after background subtraction. If figures were reconstituted from images of blots, lanes from the same blot are shown in one panel of a figure, even if they were in a different order on the original blot (separated by dotted lines). Data from different blots are shown in separate figure parts.

Yeast split ubiquitin assay

For direct interaction assays of membrane proteins in yeast, the split-ubiquitin system (SUS) was used (Grefen et al., 2009). For bridge assays for the detection of tripartite complexes, the split-ubiquitin bridge assays (SUB) was used as described by Grefen (2014).

BiFC assays

Fusion proteins for BRI1 and BIR3 with the N- or C-terminal part of YFP were transiently expressed in *N. benthamiana* (Grefen et al., 2010). Fluorescence of reconstituted YFP protein was visualized by confocal laser scanning microscopy with a TCS SP2 confocal laser-scanning microscope three days after infiltration.

BL assays

Root and hypocotyl growth assays after treatment with BL and BRZ were performed as described in Halter et al. (2014b) and Albrecht et al. (2008). BL was applied in protein release studies for 90 min before addition of MAMPs because of the different timing of BL- and MAMP-induced responses (Wang et al., 2008; Schulze et al., 2010).

Statistical Methods

Statistical significance between two samples was tested with Student´s t-test, while statistical significance between groups was analyzed using one-way ANOVA combined with Tukey's honest significant difference (HSD) test. Significant differences are indicated with different letters ($p < 0.05$); detailed ANOVA results are given in Supplemental Table 3.

Accession numbers

Sequence data from this article can be found in TAIR under the following accession numbers: BIR3: At1g27190; BIR2: At3g28450; BIR1: At3g48380; BAK1/SERK3: At4g33430; BKK1/SERK4: At2g13790; BRI1: At4g39400; FLS2: At5g46330; EFR: At5g20480; PEPR1: At1g73080

Supplemental data

Supplemental Figure 1. Expression and subcellular localization of BIR3 protein

Supplemental Figure 2. Domain structure of BIR3

Supplemental Figure 3. BIR3 is kinase inactive and is weakly phosphorylated by BAK1 and BRI1 *in vitro*

Supplemental Figure 4. Expression of BIR3 in overexpression lines

Supplemental Figure 5. Quantitative effects of BIR3 expression and functional impact of tagged versions

Supplemental Figure 6. BL and BRZ responses in *BIR3* overexpressing plants and mutants

Supplemental Figure 7. *BIR3* overexpressing plants are less sensitive to flg22 than wild type and all tagged versions of BIR3 are functional

Supplemental Figure 8. BIR3 co-immunoprecipitates with BRI1-FLAG *in vivo*

Supplemental Figure 9. Expression of BIR3 in *BRI1-GFP*-expressing plants

Supplemental Figure 10. Split ubiquitin (bridge) assays and expression controls

Supplemental Figure 11. Characterization of *BIR3* T-DNA insertion lines

Supplemental Figure 12. ROS production in *bir3* mutants and in the complemented lines after PAMP treatment

Supplemental Figure 13. BL responsive gene expression in *bir3* mutants

Supplemental Figure 14. *bir3* mutants are not affected in their resistance to *Pto* DC3000 or *Alternaria brassicicola*

Supplemental Figure 15. *bak1 bir3* double mutants can be complemented by expression of BAK1 or BIR3

Supplemental Figure 16. BIR3 interacts with all SERKs *in vitro*

Supplemental Figure 17. Complementation of *BIR3* overexpression phenotypes by *BAK1* or *BRI1* overexpression

Supplemental Figure 18. BIR3 inhibits BL signaling in the absence of BAK1

Supplemental Figure 19. Transcript levels of *BAK1* and *BKK1* in Col-0 and *bir3* mutant lines

Supplemental Table 1. Peptides of BIR3 (At1g27190) identified in BRI1-FLAG IP

Supplemental Table 2. Primers used in this study

Supplemental Table 3. ANOVA results

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AUTHOR CONTRIBUTIONS

JI TH SH SS SM NS RM SP MW YY WD MS CZ MBG performed research, and analyzed data; **SC SV FT** designed research; **XW** designed research, performed research and analyzed data; **BK** designed research, analyzed data and wrote the paper.

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Figure Legends

Figure 1: Overexpression of *BIR3* leads to BL insensitivity

(A) Photograph of representative morphological phenotypes of 6-week-old Col-0, *bak1-4* and two independent lines of hemizygous (+/-) and homozygous (+/+) *Pro*35S:BIR3 plants. The scale bar on the left represents 1 cm. **(B)** Rosette diameter of lines shown in A. Results are means ± SD. **(C)** Col-0 and *Pro*35S:BIR3-FLAG lines were grown vertically for 5 days in the dark on ½ MS agar plates supplemented or not with the indicated concentrations of 24-Epi-Brassinolide (BL). Hypocotyl length was measured and presented as mean ± SE (n=16). **(D)** Seedlings of the indicated genotypes were treated with 1 µM 24-Epi-BL. Phosphorylation of BES1 was detected as a size shift on protein gel blots probed with α-BES1 antibodies. Amounts of detected proteins were quantified relative to the unphosphorylated BES1 in Col-0. The relative expression level of *CPD* **(E)** and *DWF4* **(F)** in Col-0 and *BIR3*-overexpression seedlings. Relative expression level of *CPD* and *DWF4* was measured by quantitative RT-PCR with *ACTIN2* used as the reference gene. The mRNA used for reverse transcription was extracted from 14-day-old seedlings grown on ½ MS medium with or without 1 µM 24-epiBL treated for 1 h. Data are means ± SD. Different letters indicate significant differences according to one-way ANOVA and Tukey's HSD test ($p<0.05$). The experiments were repeated at least three times with similar results.

Figure 2: BIR3 overexpressing plants are insensitive to flg22 treatment and show higher symptom development after *Alternaria brassicicola* infection than wild type

(A) ROS production was measured as relative light units (RLU) in a luminol based assay. Leaf pieces of Col-0, *bak1-4* and hemizygous *Pro*35S:BIR3 plants were elicited with 100 nM

flg22 and ROS production was measured over a period of 30 min. Values are mean ± SE (n=9). **(B)** *FRK1* marker gene expression in Col-0, *bak1-4* and homozygous *Pro*35S:BIR3 plants was measured by qRT-PCR analysis 3 hours after flg22 treatment. *FRK1* expression was normalized to *EF1α* and plotted relative to the untreated Col-0 control. Results are mean ± SE (n=8). **(C)** The indicated Arabidopsis lines were infiltrated with 10^4 cfu/ml of the virulent bacterial pathogen *Pto* DC3000. Growth of bacteria was monitored after 0, 2 and 4 days. Results are mean ± SE (n=8). No significant differences according to ANOVA analysis.
(D) Photograph of representative leaves of five-week old plants of the indicated genotypes 12 days after infection with the necrotrophic fungus *Alternaria brassicicola*. **(E)** Bonitation of disease symptoms 7 and 10 days after infection of the lines shown in (D). Shown is the disease index as mean ± SE (n=12).
Different letters indicate significant differences according to one-way ANOVA and Tukey's HSD test ($p<0.05$). All experiments were repeated at least three times with similar results.

Figure 3: BIR3 interacts with ligand-binding receptors from different pathways
(A,D,E) *Pro*35S:BIR3-YFP and other indicated constructs were transiently expressed in *N. benthamiana* leaves and immunoprecipitation (IP) was performed with GFP-trap beads. Precipitated BIR3 and co-immunoprecipitated proteins were detected with α-GFP and antibodies against the tag of the respective protein. Protein input is shown by protein gel blot analysis (abbreviated WB) of protein extracts before IP and antibodies against the respective tags. Coomassie brilliant blue (CBB) staining shows protein loading.
(A) Co-IP experiments were performed with BIR3-YFP and BRI1-HA.
(B) *Pro*35S:BIR3-GFP was stably expressed in Arabidopsis and immunoprecipitated with a GFP antibody. Co-immunoprecipitated endogenous BRI1 was detected with BRI1-specific antibodies. Protein input is shown by protein gel blot analysis of protein extracts before IP and antibodies against BRI1 or GFP. Coomassie brilliant blue (CBB) staining shows protein loading.
(C) Morphological phenotypes of plants stably expressing *Pro*35S:BIR3-FLAG in the Col-0 or *Pro*35S:BRI1-GFP background. Numbers indicate line numbers.
(D) Co-IP experiment with BIR3-YFP and FLS2-Myc.
(E) Co-IP experiments with BIR3-YFP and EFR-Myc or PEPR1-Myc.
All experiments were repeated at least three times with similar results

Figure 4: BIR3 directly interacts with BRI1 independently of BAK1
(A) Split ubiquitin system (SUS) yeast growth assays containing the two indicated proteins fused to the N- and C-terminal parts of ubiquitin were performed (ev = empty vector). Yeast was grown at three different 1 to 10 dilutions on medium selecting for vector transformation

(CSM-Leu$^-$, Trp$^-$) and selecting for interaction (CSM-Leu$^-$, Trp$^-$, Ade$^-$, His$^-$, with two different methionine concentrations). Growth was monitored after 1 day for the vector-selective control plates and after 3 days for the interaction plates. **(B)** Bimolecular fluorescence complementation analyses were performed with BIR3 fused to the C-terminal part of YFP (BIR3-cYFP) and BRI1 fused to the N-terminal part of YFP (BRI1-nYFP). As controls, both proteins were combined with the respective YFP part alone. The proteins were transiently expressed in *N. benthamiana* and fluorescence was visualized by confocal laser scanning microscopy. Bar size 100 µm. **(C, D)** Split ubiquitin bridge (SUB) assays containing the indicated two proteins of interest fused to the N- and C-terminal parts of ubiquitin and an additional "bridge" protein (ev = empty vector). Yeast was grown as described in (A). **(E)** Co-IP was performed with Arabidopsis seedlings expressing BIR3-FLAG in the *bak1-4* background and with the *bir3-2* mutant as controls. Immunoprecipitation (IP) was performed with BRI1 antibodies. Precipitated BRI1 and co-immunoprecipitated BIR3 protein were detected with specific α-BRI1 and α-BIR3 antibodies, respectively. Protein input is shown protein gel blot analysis of protein extracts before IP detected with α-BRI1and α-BIR3 antibodies. Coomassie brilliant blue (CBB) staining shows protein loading. All experiments were repeated at least twice with identical results. Expression of the yeast-expressed proteins was verified by protein gel blot analysis (Supplemental Figure 10).

Figure 5: BIR3 regulates receptor complex formation

Arabidopsis seedlings of the indicated genotypes were treated for 5 min with (**A)** 1 µM flg22 or 1 µM *At*Pep1 or a mix of 1 µM flg22, 1 µM elf18, 1 µM *At*Pep1 and 90 min before exposure to the other ligands with 100 nM 24-Epi-BL and water as control (-), or in (**B)** with 100 nM 24-Epi-BL. IP was performed with GFP-trap beads. Precipitated BIR3-GFP and co-immunoprecipitated BAK1 or BRI1 were detected with specific α-GFP, α-BRI1 and α-BAK1 antibodies, respectively. Protein input is shown by protein gel blot analysis of protein extracts before IP and α-BAK1, α-BRI1 and α-GFP antibodies. Coomassie brilliant blue (CBB) staining shows protein loading. **(C)** Arabidopsis seedlings of the indicated genotypes were treated for 5 min with 1 µM flg22 (+) or H_2O (-). IP was performed with specific α-BAK1 antibodies. Precipitated BAK1 and co-immunoprecipitated FLS2 were detected with specific α-BAK1 and α-FLS2 antibodies, respectively. Protein input is shown by protein gel blot analysis of protein extracts before IP and specific α-BAK1 and α-FLS2 antibodies. Coomassie brilliant blue (CBB) staining shows protein loading. **(D)** BIR3-FLAG and BRI1-GFP were stably expressed in *Arabidopsis* leaves and IP was performed with GFP antibodies and protein A. Precipitated BRI1 and co-immunoprecipitated proteins were detected with α-GFP, α-FLAG and BAK1-specific antibodies. Protein input is shown by protein gel blot analysis of protein extracts before IP and antibodies against the respective

tags or BAK1. All experiments were repeated at least three times with similar results. Quantification results relative to the Col-0 controls are given as number inserts in the figures

Figure 6: *bir3* mutants show a weak MAMP and BL phenotype (A) Fresh-weight of 12-day-old Col-0, *bir3-1* and *bir3-2* seedlings grown for 7 days with or without 100 nM of the indicated peptide. The line graph represents the average fresh weight ± SE (n=48), Student´s t-test *** p<0.01; ** p<0.05; * p<0.1. **(B)** ROS production was measured as relative light units (RLU) in a luminol-based assay. Leaf pieces of the indicated Arabidopsis lines were elicited with 100 nM flg22 and ROS production was measured over a period of 30 min; cBIR3-GFP is a *bir3-2* mutant complemented with the genomic coding region under its native promotor. Values are mean ± SE (n=9). **(C)** The indicated Arabidopsis lines were grown vertically for 5 days in the dark on ½ MS agar plates supplemented with the indicated concentrations of 24-Epi-BL. Hypocotyl length was measured and presented relative to untreated controls. Values are means ± SD, (n=15). **(D)** Seedlings of the indicated genotypes were treated with1 µM 24-Epi-BL. Phosphorylation of BES1 was detected as a size shift on protein gel blots probed with α-BES1 antibodies. Amounts of detected proteins were quantified relative to the unphosphorylated BES1 in Col-0. **(E)** Photograph of representative 24-day-old plants of the indicated genotypes from the top (upper panel) and the side (lower panel). **(F)** Mean plant height ± SD (n=5) of the same lines shown in (E). **(G)** Mean rosette width ± SD (n=5) of the lines shown in (E). Different letters indicate statistical differences according to one-way ANOVA and Tukey's HSD test (p<0.05). All experiments were repeated at least three times with similar results.

Figure 7: Loss of BIR3 enhances *bak1* cell death

(A) Morphological phenotypes of different allele combinations of *bak1 bir3* double mutants and the respective single mutants. **(B)** Trypan blue staining to stain dead cells in leaves of the indicated genotypes 4 days after inoculation with *Alternaria brassicicola* (+) or untreated (-). Scale bar represents 0.5 cm. **(C, D)** qRT-PCRs to analyze *PR1* and *PDF1.2* marker gene expression in leaves of untreated Arabidopsis plants of the indicated genotypes. Gene expression was normalized to the housekeeping gene *EF1α* and is plotted relative to Col-0. Results are mean ± SE (n=6). Letters indicate significant differences according to one-way ANOVA Tukey's HSD test (p<0.05). **(E, F)** Gas-chromatography-MS quantification of SA and JA content in 5 week-old leaves of untreated Arabidopsis plants of the indicated genotypes. Results are presented as mean ± SE (n=6). Letters indicate significant differences according to one-way ANOVA Tukey's HSD test (p<0.05). All experiments were repeated at least three times with similar results.

Figure 8: BIR3 stabilizes BAK1 and BKK1

(A) Protein gel blot analysis of BAK1 and FLS2 protein amounts in Col-0, *bir3-2* and *Pro*35S:BIR3. Seedlings were treated with 1µM flg22 (+) or H_2O (-) and protein amounts were detected with specific α-BAK1 and α-FLS2 antibodies, respectively. Coomassie brilliant blue (CBB) staining shows protein loading. **(B)** BKK1-Myc and BIR3-YFP constructs were transiently expressed in *N. benthamiana*. Protein gel blot analysis on total protein extracts with α-GFP and α-Myc antibodies shows BKK1-Myc and BIR3-YFP protein amounts. Coomassie brilliant blue (CBB) staining shows protein loading. **(C)** The indicated constructs were transiently expressed in *N. benthamiana* leaves and IP was performed with GFP-trap beads. Precipitated BIR3-YFP and co-immunoprecipitated BKK1-HA were detected with α-GFP and α-HA antibodies, respectively. Protein input is shown by protein gel blot (WB) analysis of protein extracts before IP and α-GFP and α-HA antibodies. Coomassie brilliant blue (CBB) staining shows protein loading. **(D)** Morphological phenotypes of different allele combinations of *bak1 bir3* double mutants and *bak1-3 bkk1* (Albrecht et al., 2008). All experiments were repeated at least three times with similar results. Quantification results relative to the Col-0 controls are given as number inserts in the figures.

Figure 9: BL and BRZ responses are enhanced in *bir3* mutants expressing BAK1

Representative photographs of seedlings of the indicated genotypes grown on ½ MS medium with BL treatment of the indicated concentrations in the light for 7 days **(A)** and in the dark for 5 days **(B)** are shown. **(C)** Root lengths and **(D)** hypocothyl length relative to untreated controls of the seedlings corresponding to **(A)** or **(B),** respectively. **(E)** Protein gel blot detection of BAK1-GFP levels in the indicated plant lines with α-GFP antibodies. CBB staining of the membrane shows protein loading. Number inserts are quantifications of the signals relative to the signal of BAK1-GFP in *bak1-4*. Representative photographs of 5-day-old seedlings of the indicated genotypes grown on ½ MS medium with BRZ **(F) or** BL **(G)** treatment of the indicated concentrations in the dark are shown. **(H) (I)** Hypocotyl lengths relative to the untreated controls of the seedlings shown in **(F)** or **(G)**, respectively. Data are means ± SD, n>=15. Bar size 10 mm. All experiments were repeated at least three times with similar results.

Figure 10: Model of BIR3 activity

BIR3 interacts with BAK1 and ligand binding receptors such as BRI1 or FLS2. BIR3 prevents the formation of the BAK1 receptor complex and thereby prevents downstream signaling activation. Once a ligand is perceived by its receptor, BIR3 is released from BAK1 and the ligand binding receptor, and the BAK1 receptor complex can form to initiate downstream

signaling. BIR3 also stabilizes the positive regulator BAK1. This effect antagonizes its negative regulatory function on complex formation.

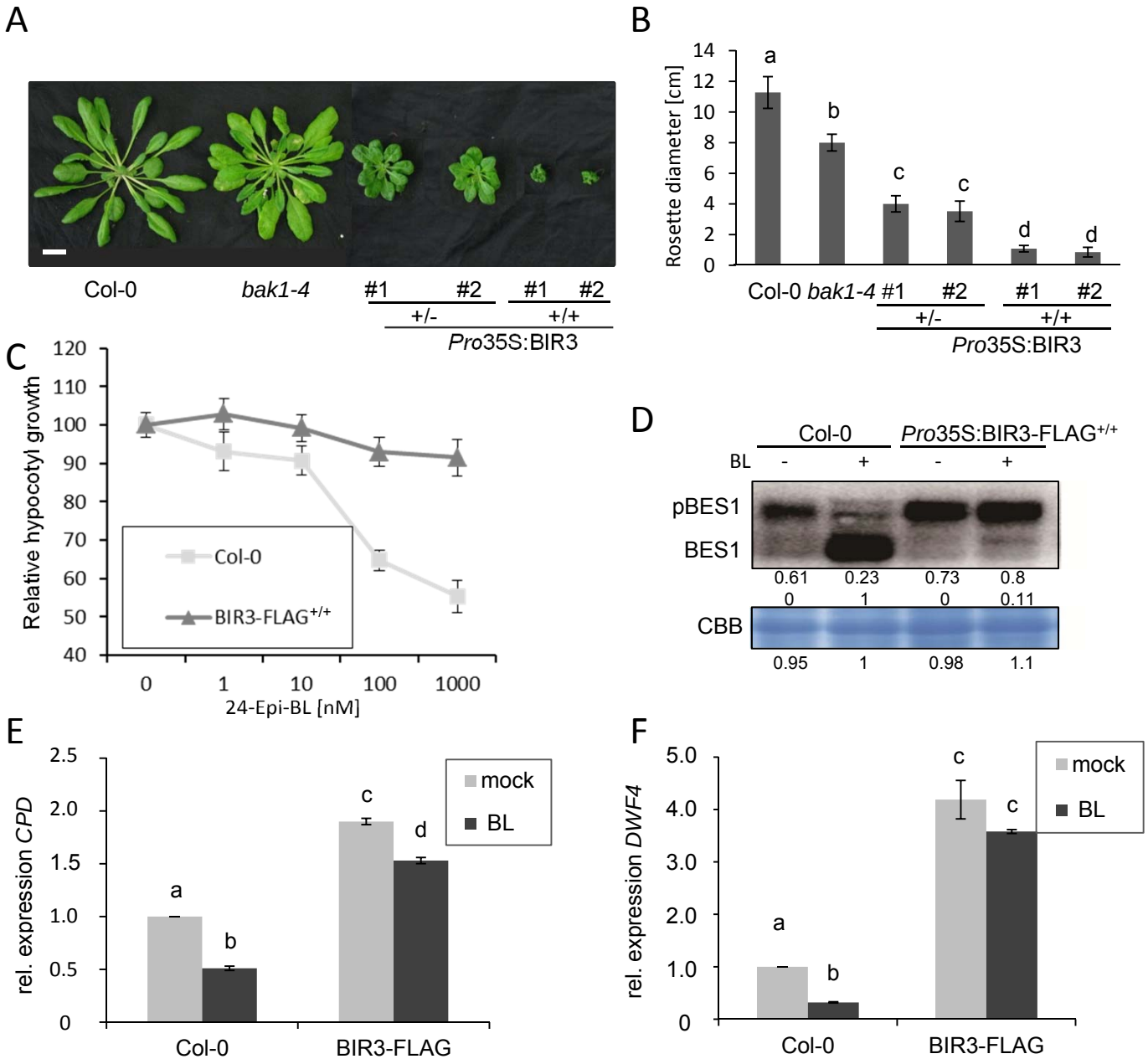

Figure 1: Overexpression of *BIR3* leads to BL insensitivity

(A) Representative pictures of the morphological phenotype of 6-week-old Col-0, *bak1-4* and two independent lines of hemizygous (+/-) and homozygous (+/+) *Pro*35S:BIR3 plants. The scale bar on the left represents 1 cm. **(B)** Rosette diameter of lines shown in A. Results are means ± SD. **(C)** Col-0 and *Pro*35S:BIR3-FLAG lines were grown vertically for 5 days in the dark on ½ MS agar plates supplemented or not with the indicated concentrations of 24-Epi-Brassinolide (BL). Hypocotyl length was measured and presented as mean ± SE (n=16). **(D)** Seedlings of the indicated genotypes were treated with 1 µM 24-Epi-BL. Phosphorylation of BES1 was detected as a size shift on Western blots probed with α-BES1 antibodies. Amounts of detected proteins were quantified relative to the unphosphorylated BES1 in Col-0. The relative expression level of *CPD* **(E)** or *DWF4* **(F)** in Col-0 and *BIR3*-overexpression seedlings. Relative expression level of *CPD* and *DWF4* was measured by quantitative RT-PCR with *ACTIN2* used as the reference gene. The mRNA used for reverse transcript were extract from 14-day-old seedlings grown on ½ MS medium with or without 1 µM 24-epiBL treated for 1 h. Data are means ± SD. Different letters indicate significant differences according to one-way ANOVA and Tukey's HSD test ($p<0.05$). The experiments were repeated at least three times with similar results.

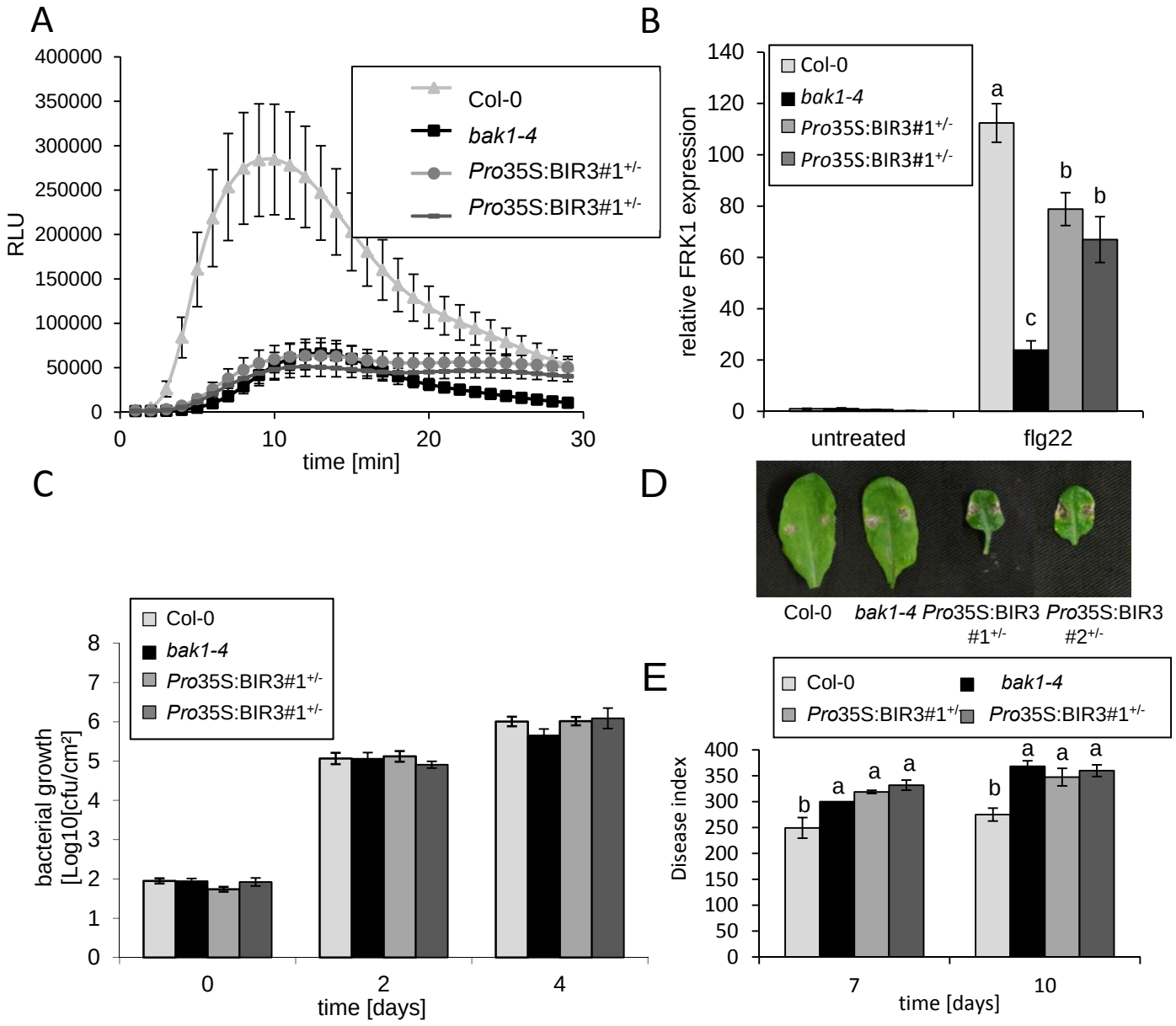

Figure 2: BIR3 overexpressing plants are insensitive to flg22 treatment and show higher symptom development after *Alternaria brassicicola* infection

(A) ROS production was measured as relative light units (RLU) in a luminol based assay. Leaf pieces of Col-0, *bak1-4* and hemizygous *Pro*35S:BIR3 plants were elicited with 100 nM flg22 and ROS production was measured over a period of 30 min. Values are mean ± SE (n=9). **(B)** *FRK1* marker gene expression in Col-0, *bak1-4* and homozygous *Pro*35S:BIR3 plants was measured by qRT-PCR analysis 3 hours after flg22 treatment. *FRK1* expression was normalized to *EF1α* and plotted relative to Col-0 untreated. Results are mean ± SE (n=8). **(C)** Indicated Arabidopsis lines were infiltrated with 10^4 cfu/ml of the virulent bacterial pathogen *Pto* DC3000. Growth of bacteria was monitored after 0, 2 and 4 days. Results are mean ± SE (n=8). No significant differences according to ANOVA analysis.
(D) Representative pictures of leaves of five-week old plants of the indicated genotypes 12 days after infection with the necrotrophic fungus *Alternaria brassicicola*. **(E)** Bonitation of disease symptoms 7 and 10 days after infection of the lines shown in (D). Shown is the disease index as mean ± SE (n=12).
Different letters indicate significant differences according to one-way ANOVA and Tukey's HSD test ($p<0.05$). All experiments were repeated at least three times with similar results.

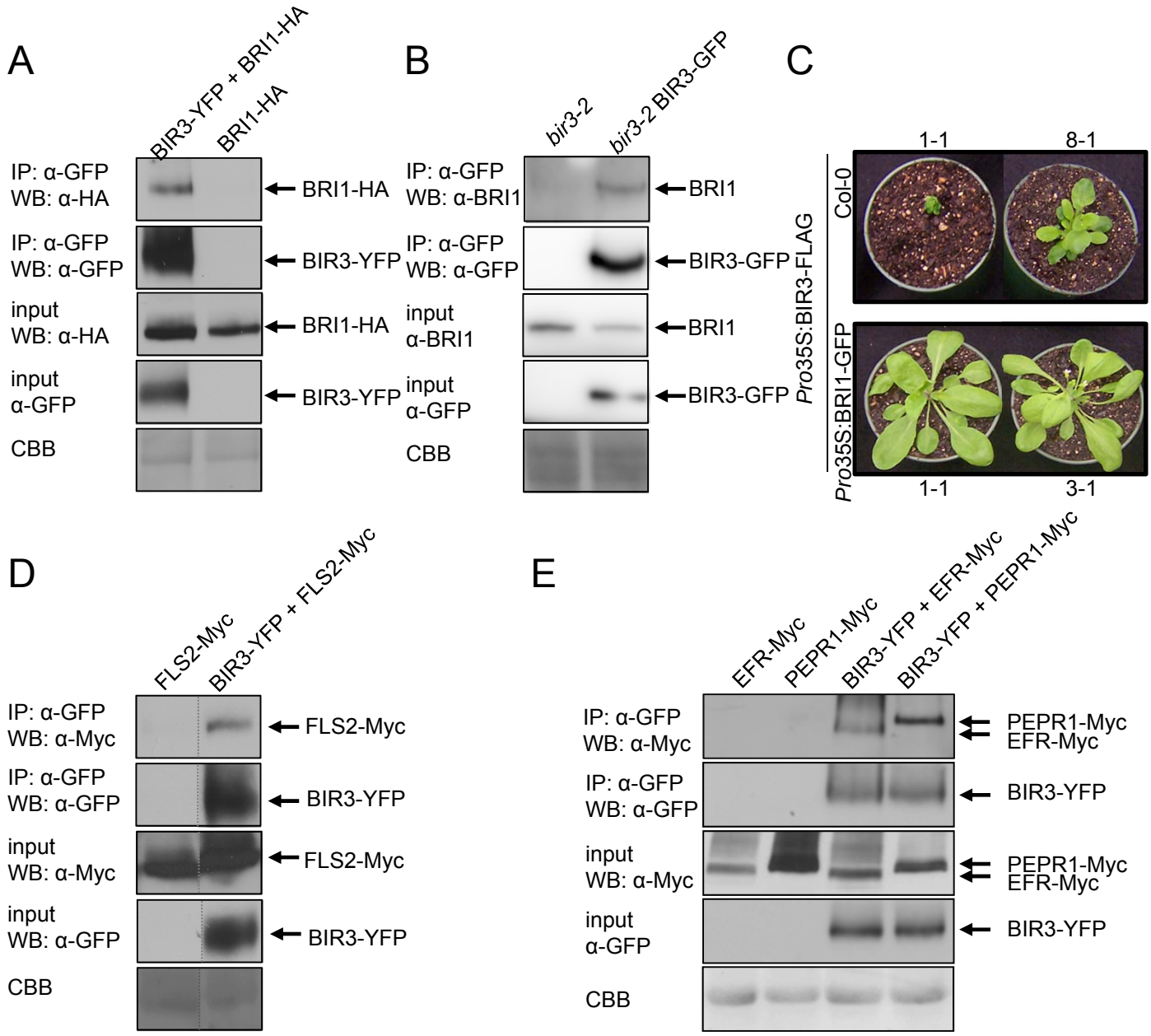

Figure 3: BIR3 interacts with ligand-binding receptors from different pathways
(A,D,E) *Pro*35S:BIR3-YFP and other indicated constructs were transiently expressed in *N. benthamiana* leaves and IP was performed with GFP-trap beads. Precipitated BIR3 and co-immunoprecipitated proteins were detected with α-GFP and antibodies against the tag of the respective protein. Protein input is shown by Western blot analysis of protein extracts before IP and antibodies against the respective tags. Coomassie brilliant blue (CBB) staining shows protein loading.
(A) Co-IP experiments were performed with BIR3-YFP and BRI1-HA.
(B) 35S-BIR3-GFP was stably expressed in Arabidopsis and immunoprecipitated with a GFP antibody. Co-immunoprecipitated endogenous BRI1 was detected with BRI1 specific antibodies. Protein input is shown by Western blot analysis of protein extracts before IP and antibodies against BRI1 or GFP. Coomassie brilliant blue (CBB) staining shows protein loading.
(C) Morphological phenotype of plants stably expressing *Pro*35S:BIR3-FLAG in Col-0 or *Pro*35S:BRI1-GFP background.
(D) Co-IP experiment with BIR3-YFP and FLS2-Myc.
(E) Co-IP experiments with BIR3-YFP and EFR-Myc or PEPR1-Myc.
All experiments were repeated at least three times with similar results.

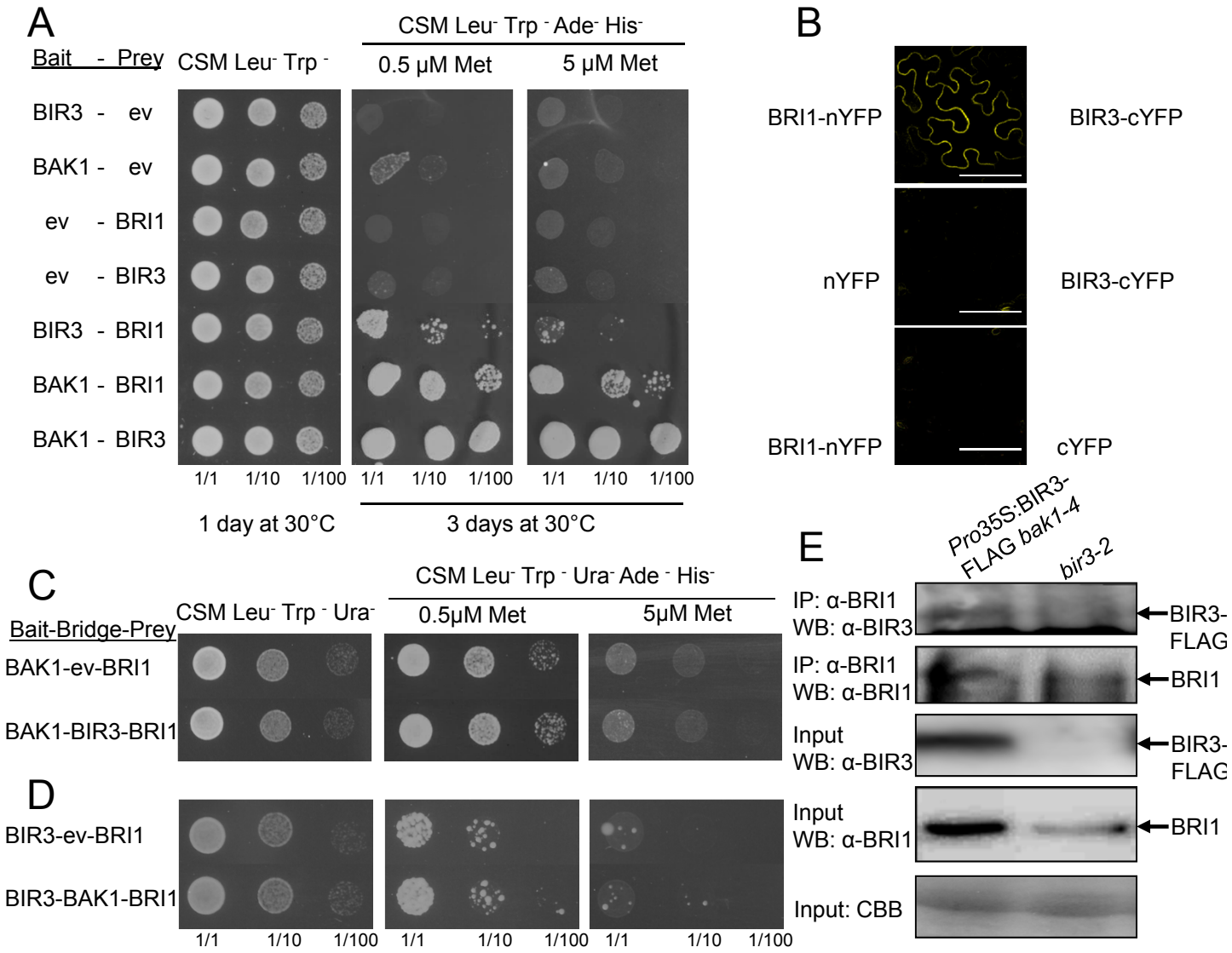

Figure 4: BIR3 directly interacts with BRI1 independent of BAK1

(A) Split ubiquitin system (SUS) yeast growth assays containing the indicated two proteins fused to the N- and C-terminal parts of ubiquitin were performed (ev = empty vector). Yeast was grown at three different 1 to 10 dilutions on medium selecting for vector transformation (CSM-Leu$^-$, Trp$^-$) and selecting for interaction (CSM-Leu$^-$, Trp$^-$, Ade$^-$, His$^-$, with two different methionine concentrations). Growth was monitored after 1 day for the vector-selective control plates and after 3 days for the interaction plates, respectively. **(B)** Bimolecular fluorescence complementation analyses were performed with BIR3 fused to the C-terminal part of YFP (BIR3-cYFP) and BRI1 fused to the N-terminal part of YFP (BRI1-nYFP). As controls both proteins were combined with the respective YFP part alone. The proteins were transiently expressed in *N. benthamiana* and fluorescence was visualized by confocal laser scanning microscopy. Bar size 100 µm. **(C, D)** Split ubiquitin bridge (SUB) assays containing the indicated two proteins of interest fused to the N- and C-terminal parts of ubiquitin and an additional "bridge" protein (ev = empty vector). Yeast was grown as described in (A). **(E)** Co-IP was performed with Arabidopsis seedlings expressing BIR3-FLAG in *bak1-4* background and *bir3-2* mutants as controls. IP was performed with BRI1 antibodies. Precipitated BRI1 and co-immunoprecipitated BIR3 protein were detected with specific α-BRI1 and α-BIR3 antibodies, respectively. Protein input is shown by Western blot analysis of protein extracts before IP detected with α-BRI1and α-BIR3 antibodies. Coomassie brilliant blue (CBB) staining shows protein loading. All experiments were repeated at least two times with identical results. Expression of the yeast expressed proteins was verified by Western blot analysis (Supplemental Figure S10).

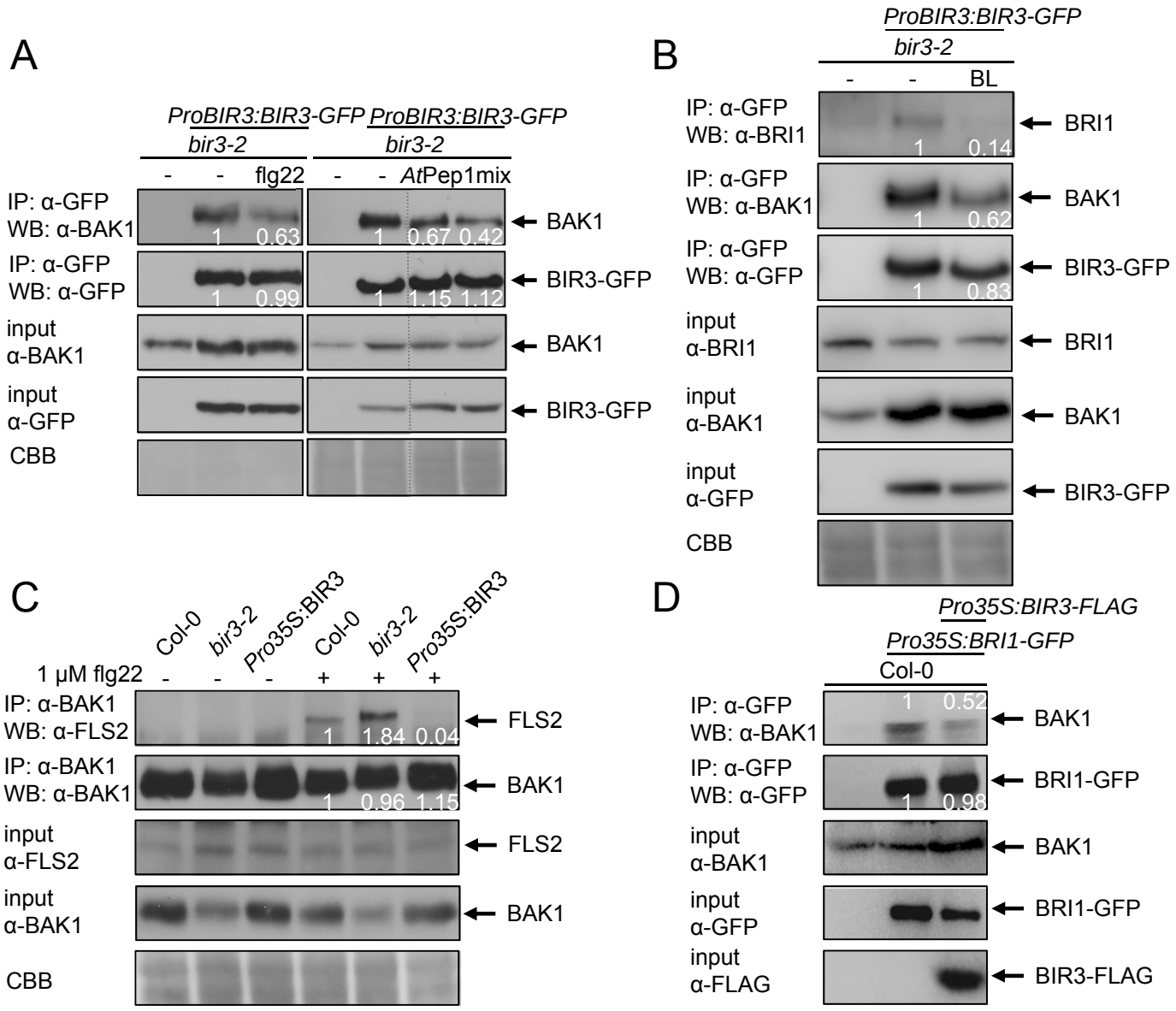

Figure 5: BIR3 regulates receptor complex formation
Arabidopsis seedlings of the indicated genotypes were treated for 5 min with (**A)** 1 μM flg22 or 1 μM *At*Pep1 or a mix of 1 μM flg22, 1 μM elf18, 1 μM *At*Pep1 and 90 min before exposure to the other ligands with 100 nM 24-Epi-BL and water as control (-), or in (**B)** with 100 nM 24-Epi-BL. IP was performed with GFP-trap beads. Precipitated BIR3-GFP and co-immunoprecipitated BAK1 or BRI1 were detected with specific α-GFP, α-BRI1 and α-BAK1 antibodies, respectively. Protein input is shown by Western blot analysis of protein extracts before IP and α-BAK1, α-BRI1 and α-GFP antibodies. Coomassie brilliant blue (CBB) staining shows protein loading. **(C)** Arabidopsis seedlings of the indicated genotypes were treated for 5 min with 1 μM flg22 (+) or H_2O (-). IP was performed with specific α-BAK1 antibodies. Precipitated BAK1 and co-immunoprecipitated FLS2 were detected with specific α-BAK1 and α-FLS2 antibodies, respectively. Protein input is shown by Western blot analysis of protein extracts before IP and specific α-BAK1 and α-FLS2 antibodies. Coomassie brilliant blue (CBB) staining shows protein loading. **(D)** BIR3-FLAG and BRI1-GFP were stably expressed in *Arabidopsis* leaves and IP was performed with GFP antibodies and protein A. Precipitated BRI1 and co-immunoprecipitated proteins were detected with α-GFP, α-FLAG and BAK1 specific antibodies. Protein input is shown by Western blot analysis of protein extracts before IP and antibodies against the respective tags or BAK1. All experiments were repeated at least three times with similar results. Quantification relative to the Col-0 controls are given as number inserts in the figures.

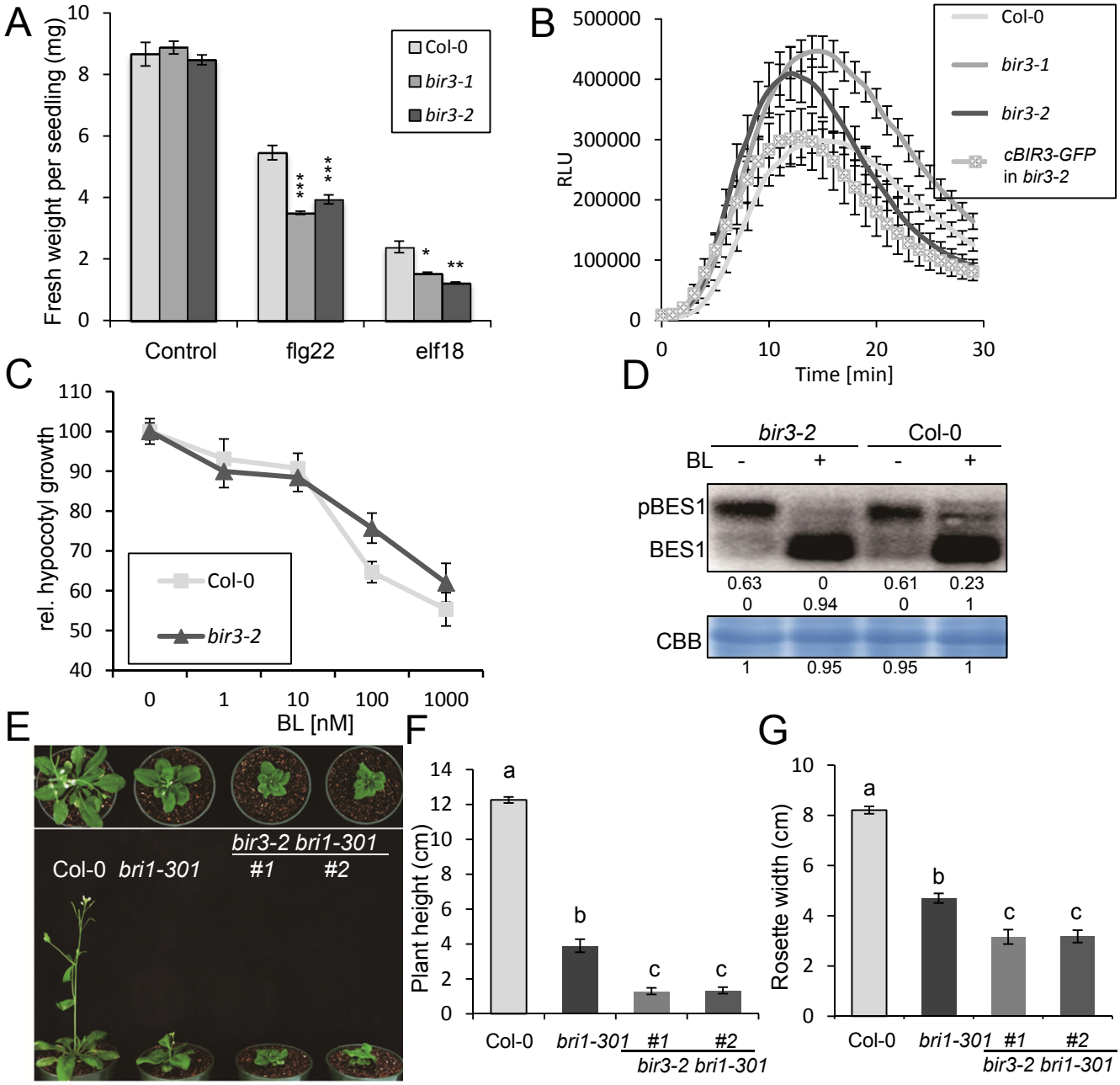

Figure 6: *bir3* mutants show a weak MAMP and BL phenotype (A) Fresh-weight of 12-day-old Col-0, *bir3-1* and *bir3-2* seedlings grown for 7 days with or without 100 nM of the indicated peptide. The line graph represents the average fresh-weight ± SE (n=48), Student´s t-test *** p<0.01; ** p<0.05; * p<0.1. **(B)** ROS production was measured as relative light units (RLU) in a luminol based assay. Leaf pieces of the indicated Arabidopsis lines were elicited with 100 nM flg22 and ROS production was measured over a period of 30 min; cBIR3-GFP is a *bir3-2* mutant complemented with the genomic coding region under its native promotor. Values are mean ± SE (n=9). **(C)** Indicated Arabidopsis lines were grown vertically for 5 days in the dark on ½ MS agar plates supplemented with the indicated concentrations of 24-Epi-BL. Hypocotyl length was measured and presented relative to untreated controls. Values are means ± SD, (n=15). **(D)** Seedlings of the indicated genotypes were treated with 1 µM 24-Epi-BL. Phosphorylation of BES1 was detected as a size shift on Western blots probed with α-BES1 antibodies. Amounts of detected proteins were quantified relative to the unphosphorylated BES1 in Col-0. **(E)** Representative pictures of the 24 day-old plants of the indicated genotypes from the top (upper panel) and the side (lower panel). **(F)** Mean plant height ± SD (n=5) of the same lines shown in (E). **(G)** Mean rosette width ± SD (n=5) of the lines shown in (E). Different letters indicate statistical differences according to one-way ANOVA and Tukey's HSD test (p<0.05). All experiments were repeated at least three times with similar results.

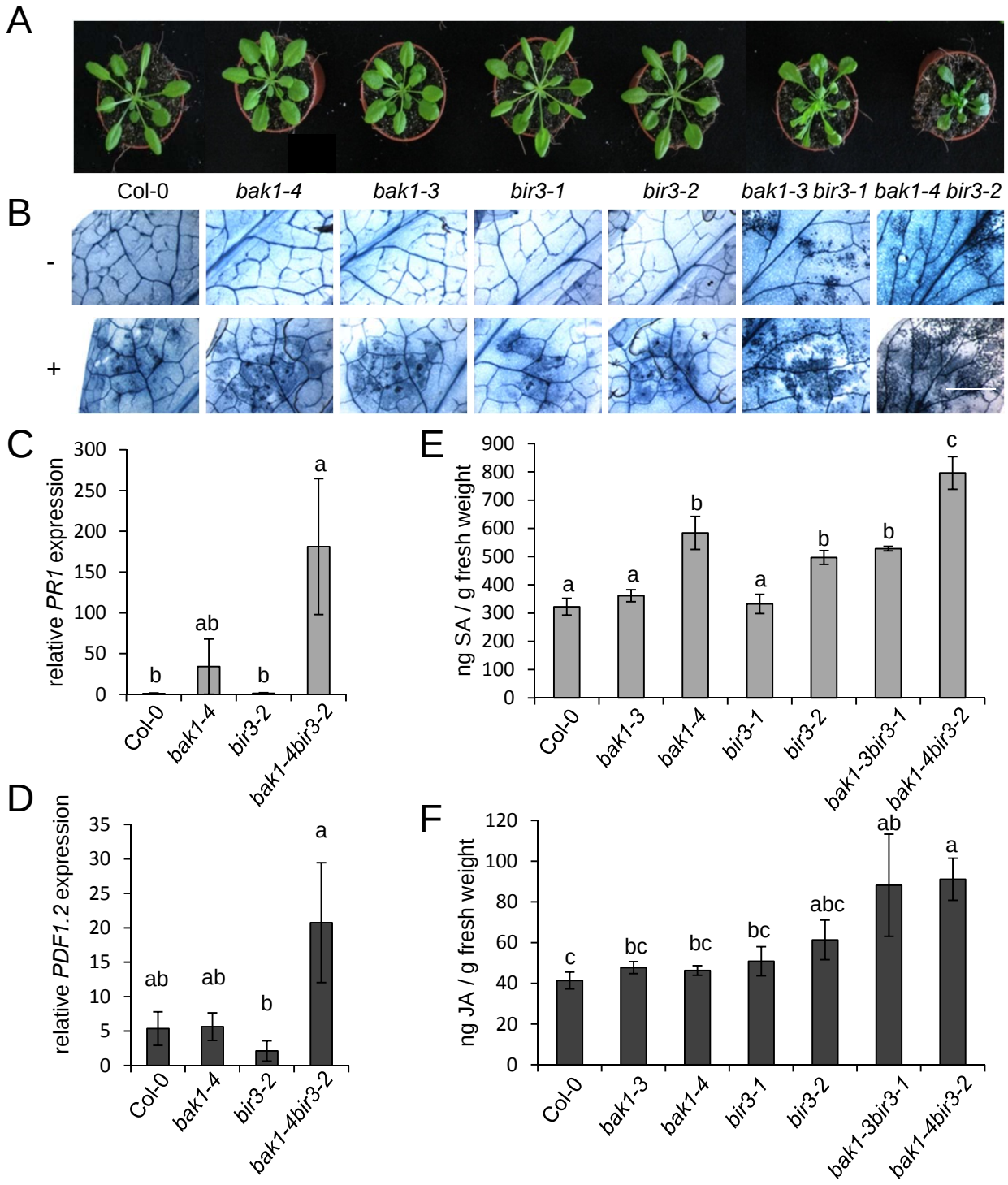

Figure 7: Loss of BIR3 enhances *bak1* cell death

(A) Morphological phenotype of different allele combinations of *bak1 bir3* double mutants and the respective single mutants. **(B)** Trypan blue staining to stain dead cells in leaves of the indicated genotypes 4 days after inoculation with *Alternaria brassicicola* (+) or untreated (-). Scale bar represents 0.5 cm. **(C, D)** qRT-PCRs to analyze *PR1* and *PDF1.2* marker gene expression in leaves of untreated Arabidopsis plants of the indicated genotypes. Gene expression was normalized to the housekeeping gene *EF1α* and is plotted relative to Col-0. Results are mean ± SE (n=6). Letters indicate significant differences according to one-way ANOVA Tukey's HSD test ($p<0.05$). **(E, F)** Gas-chromatography-MS quantification of SA and JA content in 5 week-old leaves of untreated Arabidopsis plants of the indicated genotypes. Results are presented as mean ± SE (n=6). Letters indicate significant differences according to one-way ANOVA Tukey's HSD test ($p<0.05$). All experiments were repeated at least three times with similar results.

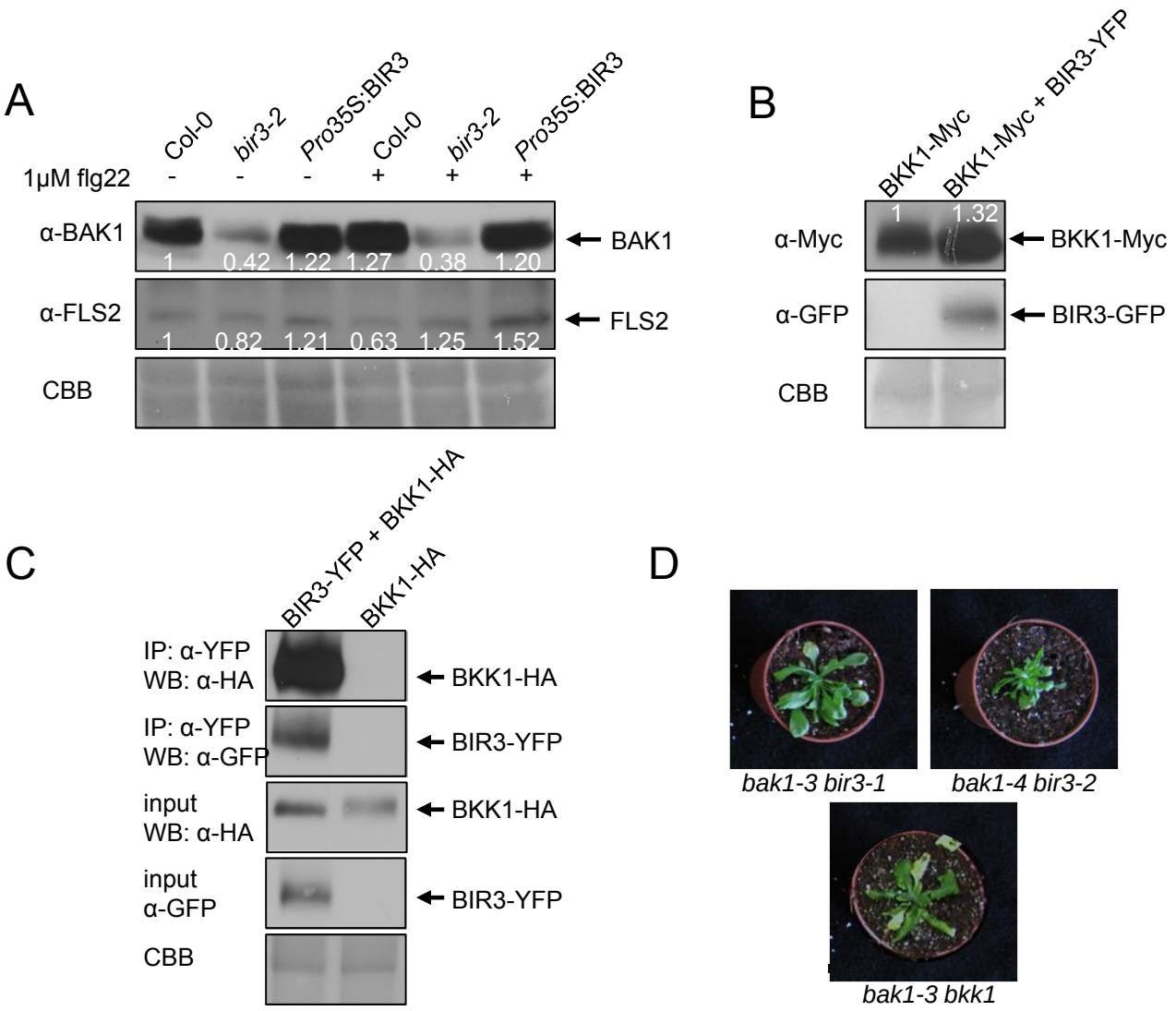

Figure 8: BIR3 stabilizes BAK1 and BKK1
(A) Western blot analysis of BAK1 and FLS2 protein amounts in Col-0, *bir3-2* and *Pro*35S:BIR3. Seedlings were treated with 1µM flg22 (+) or H_2O (-) and protein amounts were detected with specific α-BAK1 and α-FLS2 antibodies, respectively. Coomassie brilliant blue (CBB) staining shows protein loading. **(B)** BKK1-Myc and BIR3-YFP constructs were transiently expressed in *N. benthamiana*. Western-blot analysis on total protein extracts with α-GFP and α-Myc antibodies shows BKK1-Myc and BIR3-YFP protein amounts. Coomassie brilliant blue (CBB) staining shows protein loading. **(C)** Indicated constructs were transiently expressed in *N. benthamiana* leaves and IP was performed with GFP-trap beads. Precipitated BIR3-YFP and co-immunoprecipitated BKK1-HA were detected with α-GFP and α-HA antibodies, respectively. Protein input is shown by Western blot (WB) analysis of protein extracts before IP and α-GFP and α-HA antibodies. Coomassie brilliant blue (CBB) staining shows protein loading. **(D)** Morphological phenotypes of different allele combinations of *bak1 bir3* double mutants and *bak1-3 bkk1* (Albrecht et al., 2008). All experiments were repeated at least three times with similar results. Quantification relative to the Col-0 controls are given as number inserts in the figures.

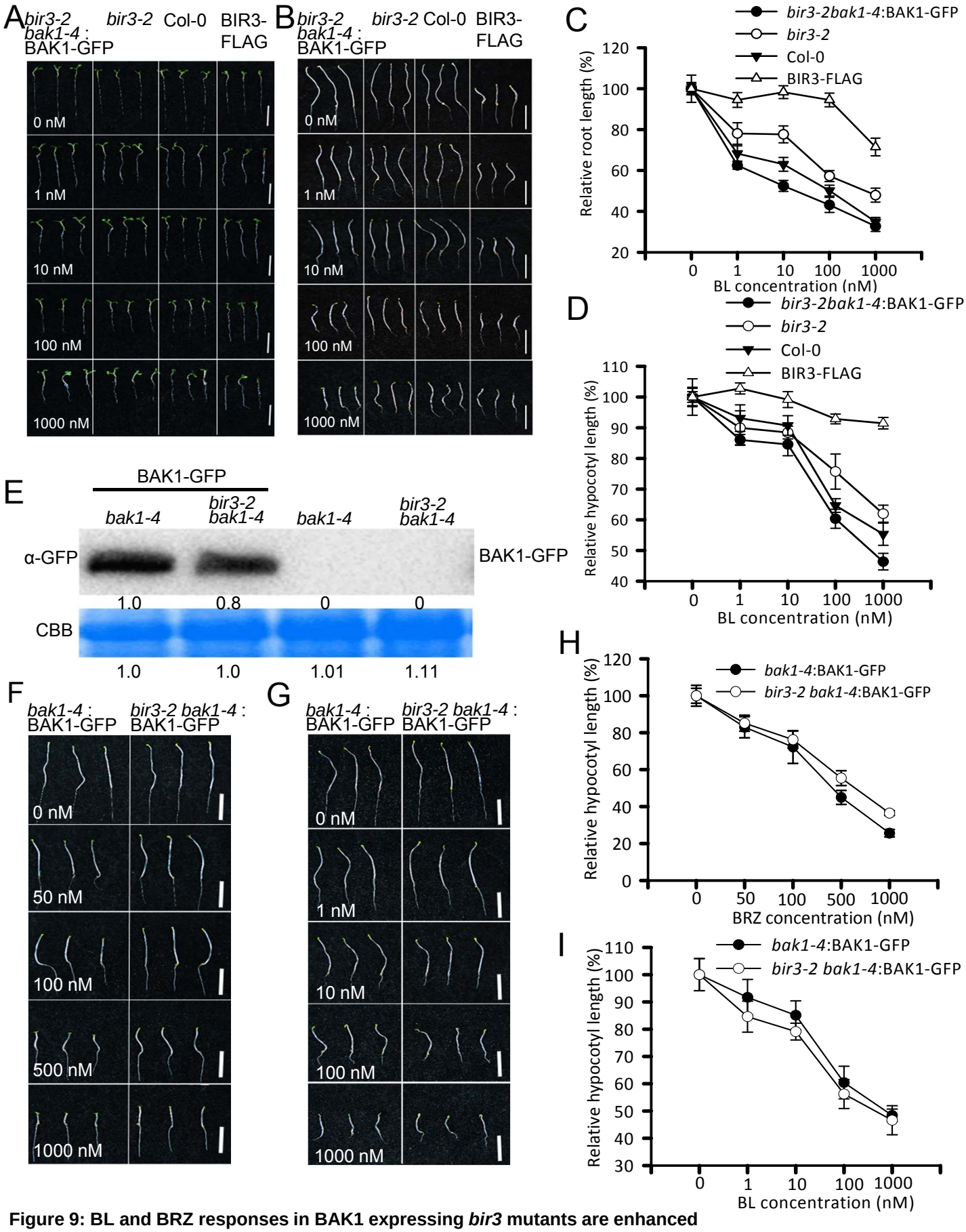

Figure 9: BL and BRZ responses in BAK1 expressing *bir3* mutants are enhanced

Representative pictures of seedlings of the indicated genotypes grown on ½ MS medium with BL treatment of the indicated concentrations in the light for 7 days **(A)** and in the dark for 5 days **(B)** are shown. **(C)** Root lengths and **(D)** hypocothyl length relative to untreated controls of the seedlings corresponding to **(A)** or **(B),** respectively. **(E)** Western blot detection of BAK1-GFP levels in the indicated plant lines with α-GFP antibodies. CBB staining of the membrane shows protein loading. Number inserts are quantifications of the signals relative to the signal of BAK1-GFP in *bak1-4*. Representative pictures of 5-day-old seedlings of the indicated genotypes grown on ½ MS medium with BRZ **(F) or** BL **(G)** treatment of the indicated concentrations in the dark are shown. **(H) (I)** Hypocotyl lengths relative to the untreated controls of the seedlings shown in **(F)** or **(G)**, respectively. Data are means ± SD, n>=15. Bar size 10 mm. All experiments were repeated at least three times with similar results.

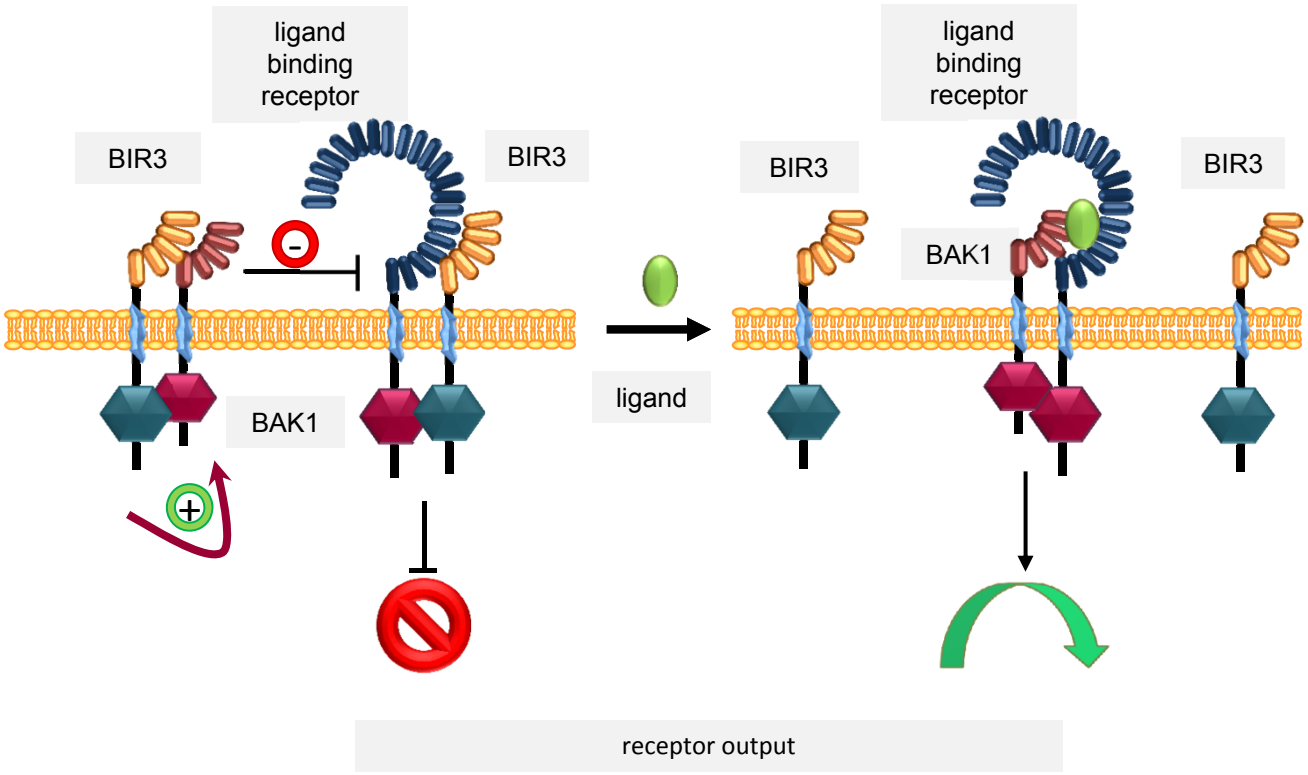

Figure 10: Model of BIR3
BIR3 interacts with BAK1 and ligand binding receptors such as BRI1 or FLS2. BIR3 prevents formation of the BAK1 receptor complex and thereby prevents downstream signaling activation. Once a ligand is perceived by its receptor, BIR3 is released from BAK1 and the ligand binding receptor, and the BAK1 receptor complex can form to initiate downstream signaling. BIR3 also stabilizes the positive regulator BAK1. This effect antagonizes its negative regulatory function on the complex formation.

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