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BAK1 is required for the attenuation of ethylene-inducing xylanase (Eix)-induced defense responses by the decoy receptor LeEix1

Maya Bar[†], Miya Sharfman[†], Mily Ron[‡] and Adi Avni[*]

Department of Plant Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel

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[*]For correspondence (fax +972 3 6410195; e-mail lpavni@post.tau.ac.il).
[†]These authors contributed equally to this work.
[‡]Present address: Section of Plant Biology and Genome Center, UC Davis, Davis, CA, 95616, USA.

SUMMARY

Elicitor recognition plays a key role in the reaction of plants to pathogens and the induction of plant defense responses. Furthermore, plant–microbe interactions involve numerous regulatory systems essential for plant defense against pathogens. Ethylene-inducing xylanase (Eix) is a potent elicitor of plant defense responses in specific cultivars of tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*). The Eix receptors (*LeEix1* and *LeEix2*) belong to a superclade of leucine-rich repeat receptor-like proteins (RLP) with a signal for receptor-mediated endocytosis, which was shown to be essential for proper induction of defense responses. Both receptors are able to bind Eix, while only LeEix2 mediates defense responses. Here we demonstrate that LeEix1 heterodimerizes with LeEix2 upon application of the Eix elicitor. We show that LeEix1 attenuates Eix-induced internalization and signaling of the LeEix2 receptor. Furthermore, we demonstrate, using yeast two-hybrid and *in planta* bimolecular fluorescence complementation assays, that the brassinosteroid co-receptor, BAK1, binds LeEix1 but not LeEix2. In BAK1-silenced plants, LeEix1 was no longer able to attenuate plant responses to Eix, indicating that BAK1 is required for this attenuation. We suggest that LeEix1 functions as a decoy receptor for LeEix2, a function which requires BAK1.

Keywords: endocytosis, elicitor, decoy receptor, plant defense, signaling.

INTRODUCTION

Leucine-rich-repeat receptor kinases (LRR-RLKs) and proteins (LRR-RLPs) have been implicated in signaling as well as defense responses in plants (Becraft, 2002; Bittel and Robatzek, 2007; Postel and Kemmerling, 2009). One such LRR-RLP is the ethylene-inducing xylanase (Eix) receptor LeEix2. The fungal protein Eix (Dean *et al.*, 1989) is a well-known protein elicitor of defense response reactions in tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*) plants (Bailey *et al.*, 1990; Avni *et al.*, 1994). Eix induces ethylene biosynthesis, electrolyte leakage, media alkalinization, expression of pathogenesis-related (PR) proteins and the hypersensitive response (HR) in specific plant species and/or varieties (Bailey *et al.*, 1990, 1992; Ron *et al.*, 2000; Elbaz *et al.*, 2002; Bargmann *et al.*, 2006). It has been shown that Eix specifically binds to the plasma membrane of responding cultivars in both tomato and tobacco (Hanania *et al.*, 1997).

In a recent work (Bar and Avni, 2009a), we have shown that Eix triggers internalization of the LeEix2 receptor. Upon application, Eix binds the LeEix2 receptor on the outside of the plasma membrane (Hanania *et al.*, 1997; Ron and Avni, 2004). This binding allows the entry of LeEix2 into the cell.

The FYVE domain has been reported to localize to endosomes in mammalian cells (Stenmark *et al.*, 1996) as well as plant cells (Jensen *et al.*, 2001; Heras and Drobak, 2002; Voigt *et al.*, 2005). Ten to 15 min after Eix application, the GFP-tagged LeEix2 receptor can be seen throughout the cell on FYVE endosomes (Bar and Avni, 2009a), whereas in untreated leaves GFP-tagged LeEix2 did not appear co-localized with the FYVE marker. In untreated leaves GFP-LeEix2 was localized to the plasma membrane (Bar and Avni, 2009a). The internalization of LeEix2 is required for induction of defense responses (Bar and Avni, 2009a,b; Bar *et al.*, 2009).

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Characterization of the *LeEix* locus of *S. lycopersicum* by map-based cloning led to the identification of two LRR-RLPs, *LeEix1* and *LeEix2*. While both receptors bind Eix and contain a YXXϕ endocytosis motif in their cytoplasmic region, only *LeEix2* transmits the signal that results in Eix-derived defense responses (Ron and Avni, 2004).

Flagellin sensitive 2 (FLS2), which recognizes bacterial flagellin and the flagellin-derived peptide flg22 (Felix *et al.*, 1999; Gomez-Gomez *et al.*, 1999; Gomez-Gomez and Boller, 2000), has been the subject of many works in connection with plant defense responses. Recognition of flg22 by FLS2 leads to a response which includes generation of reactive oxygen species (ROS), mitogen-activated protein kinase (MAPK) activation, ethylene production and induction of gene transcription (Felix *et al.*, 1999; Asai *et al.*, 2002; Zipfel *et al.*, 2004). Mutations in FLS2 compromised the ability of the plant to mount an efficient defense against bacterial pathogens (Zipfel *et al.*, 2004; Robatzek *et al.*, 2006). Further, FLS2 internalization was also found to be important for signaling (Robatzek *et al.*, 2006).

It was found that FLS2 is in a complex with the brassinosteroid co-receptor BAK1, and cannot mediate proper flagellin-derived signaling in BAK1-silenced plants (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). BAK1, also known as SERK3, a receptor kinase originally isolated in connection with the brassinosteroid receptor BRI1 which it positively regulates (Hecht *et al.*, 2001; Li *et al.*, 2002; Chinchilla *et al.*, 2009; Postel *et al.*, 2010), has since been found to be involved in plant defense. BAK1 influences endocytosis of both BRI1 and FLS2 (Russinovaa *et al.*, 2004; Chinchilla *et al.*, 2007; Geldner *et al.*, 2007). Interestingly, Arabidopsis and *Nicotiana benthamiana* plants silenced in or lacking the BAK1 protein show reduced flagellin sensitivity (Chinchilla *et al.*, 2007; Heese *et al.*, 2007); it has been suggested that BAK1 may act as a signaling partner for FLS2 (Chinchilla *et al.*, 2009).

It has also been reported recently that BAK1 may be involved in the resistance of tomato cultivars to *Verticillium dahliae* via the plant LRR receptor *Ve1*, an LRR-RLP, as tomato plants silenced in *BAK1* showed higher susceptibility to *Verticillium* infection (Fradin *et al.*, 2009).

In this work we show that LeEix1 and LeEix2 interact upon Eix application, and that *LeEix1* is capable of attenuating the endocytosis and signaling of *LeEix2* upon overexpression in response to Eix. Further we demonstrate that LeEix1 interacts with BAK1, and BAK1 is required for the ability of *LeEix1* to attenuate the signaling of *LeEix2*.

RESULTS

LeEix1 interacts with LeEix2 upon Eix application

The discovery that the *LeEix* tomato locus contains two genes which encode functional receptors capable of binding the Eix ligand, but that only *LeEix2* can transmit Eix-dependent signaling (Ron and Avni, 2004), was intriguing. In order to obtain insight into the importance of *LeEix1* and the mechanisms behind Eix signaling, we examined whether the two receptors interact *in planta* in the bimolecular fluorescence complementation (BiFC) system (Bracha-Drori *et al.*, 2004). Reconstitution of yellow fluorescent protein (YFP) fluorescence was examined by transient co-expression of *LeEix1* and *LeEix2*. Cells co-expressing YFP N-terminal half (YN)-LeEix1 and YFP C-terminal half (YC)-LeEix2 showed clear YFP fluorescence localized to the cell membrane after treatment with Eix. The two proteins failed to interact in the absence of Eix treatment (Figure 1a). YN-LeEix1 and YC-LeEix2 were examined for fluorescence with the complementary half of the YFP protein and the results were negative (Figure S1). Additionally, LeEix2 was found not to self-interact in the BiFC system, irrespective of the presence of Eix (Figure 1a). Moreover, we examined the interactions between the two receptors in the yeast

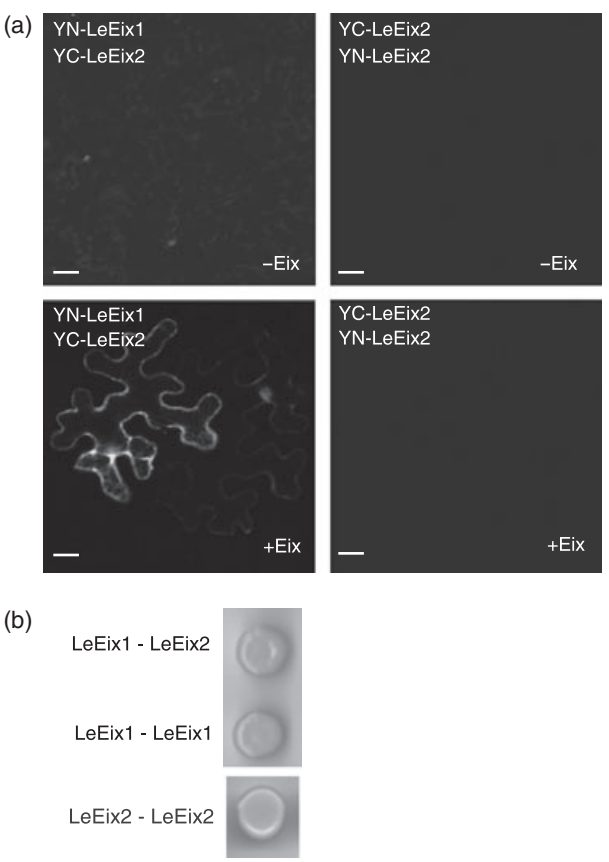

Figure 1. Interaction of LeEix1 with LeEix2.
(a) *Nicotiana benthamiana* leaves transiently expressing yellow fluorescent protein (YFP) N-terminal half (YN)-LeEix1, YFP C-terminal half (YC)-LeEix2 and YN-LeEix2 forms as indicated. Leaf sections were visualized 48 h after transformation under a Zeiss laser-scanning meta confocal microscope with or without ethylene-inducing xylanase (Eix) treatment. Bar = 20 μm.
(b) EGY48 yeast cells containing LeEix1 (in pEG202) and LeEix2 or LeEix1 (in pJG4-5) were grown on galactose medium lacking the amino acids His, Ura and Trp and supplied with X-Gal.

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two-hybrid system (Gyuris *et al.*, 1993). Yeast strains carrying *LeEix1* in the bait and *LeEix2* in the prey did not activate the LEU2 or LacZ reporter genes indicating that the two proteins do not interact (Figure 1b). Yeast strains carrying *LeEix1* or *LeEix2* in both the bait and the prey did not activate the LEU2 or LacZ reporter genes, indicating that LeEix1 and LeEix2 do not homodimerize (Figure 1b). Moreover, yeast strains carrying LeEix1 or LeEix2 in the bait and a control pray RbcL did not activate the LEU2 or LacZ reporter genes (Figure S2). Expression was dependent upon growth on galactose medium, indicating that expression of both LeEix1 and LeEix2 is required for expression of the reporter genes. We used the repression assay for DNA binding (Golemis *et al.*, 2008) to confirm the expression of LeEix1 or LeEix2 and their entrance into the nucleus (Figure S2).

LeEix1 is synthesized de novo to a greater degree than LeEix2 in response to Eix treatment

To study whether Eix treatment can induce transcription of the *LeEix* genes, total RNA was extracted from leaves at different time points after infiltration with an Eix solution. The mRNA of the *LeEix* locus increased in response to Eix treatment (Figure S3). Given the differences between *LeEix1* and *LeEix2*, in order to further examine whether one or both receptors are induced in response to Eix, and to what degree, detached *S. lycopersicum* cv. M82 leaves were incubated with or without Eix. At different time points after Eix treatment, mRNA was prepared from leaves and analyzed by semi-quantitative PCR (Figure 2). Northern blot analysis indicates that Eix induces the expression of the *LeEix* locus. Since expression of *LeEix2* was undetectable using gene-specific probes, while *LeEix1* was found to be induced with gene-specific probes, we deduced that the induction in the *LeEix* locus expression is due mostly to *LeEix1* induction (Figure S3). As can be seen in Figure 2, upon Eix treatment *LeEix1* is induced about threefold, while *LeEix2* is induced about 1.5-fold. The functionality of such an increase in *LeEix1* was unknown to us, as *LeEix2* is sufficient to transmit the signal in response to Eix. We therefore wished to examine whether LeEix1 could affect Eix signaling.

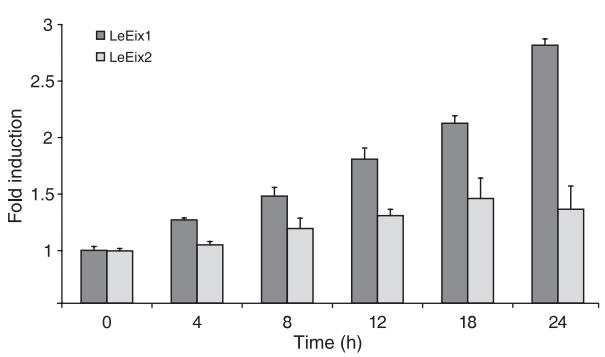

Figure 2. Induction of *LeEix* receptors mRNA upon ethylene-inducing xylanase (Eix) treatment.
Total RNA was extracted from tomato leaves at indicated time points after Eix (2.5 μg ml^{-1}) application. Semi-quantitative RT-PCR was performed on a total cDNA using specific primers to *LeEix1* and *LeEix2*. The RT-PCR products (25 cycles for *LeEix1* and 38 cycles for *LeEix2*) were separated on an agarose gel stained with ethidium bromide and quantified using ImageJ software. The RT-PCR products were calibrated against the reference tomato gene elongation factor 1A (ELF1A).
Values are the means and SE of three experiments.

LeEix1 attenuates LeEix2 endocytosis and signaling

In order to evaluate the possible effect of an increase in *LeEix1* expression upon Eix treatment, we examined the effect of transiently overexpressed Pro_{35S}*:LeEix1* on Eix signaling in *N. tabacum* cv. *Samsun* plants (which contain endogenous levels of both receptors). Transient overexpression of *LeEix1* in this system causes a delay and decrease in the level of Eix-induced defense responses. Monitoring these responses over 72 h showed significant attenuation of HR and ethylene biosynthesis after 48 h (Figure 3). This attenuation lessens 72 h after transient transformation (Figure 3). We have previously reported that Eix induces endocytosis of the LeEix2 receptor (Bar and Avni, 2009a). We further examined the effect of overexpression of LeEix1 on the endocytosis of LeEix2 in *N. benthamiana* (which does not contain endogenous LeEix receptors). We examined the co-localization of GFP-LeEix2 with the endosomal marker FYVE-DsRed (Voigt *et al.*, 2005; Bar and Avni, 2009a). We conducted 10 independent experiments in each of which 20 different cells were examined. As can be seen in Figure 3c, overexpression of LeEix1 attenuated endocytosis of LeEix2 in response to Eix treatment.

BAK1 Interacts with LeEix1 but not LeEix2

In an effort to further elucidate components of the LeEix/Eix signaling machinery, an ongoing search for interacting proteins of either receptor led us to examine BAK1. BAK1 has previously been reported to interact and/or affect additional membranal LRR-type defense receptors in plants (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Fradin *et al.*, 2009). Interestingly, we found that BAK1 interacts with the LRR domain of LeEix1 but not LeEix2 in the yeast two-hybrid system (Figure 4). Yeast strains carrying the LRR domain of LeEix1 in the bait and BAK1 in the prey grew in the absence of leucine, indicating LEU2 reporter gene activation. When grown on X-gal plates, these yeast cells were blue as a result of LacZ reporter gene activation (Figure 4). In contrast, a control yeast strain expressing the arbitrary bait, Bicoid (LexA fused to a transcriptionally inert fragment of the *Drosophila* Bicoid product), and the BAK1 prey did not activate the LEU2 or LacZ reporter genes (Figure S2). Expression was dependent upon growth on galactose medium, indicating that expression of BAK1 in the prey was required for expression of the reporter genes. Yeast strains

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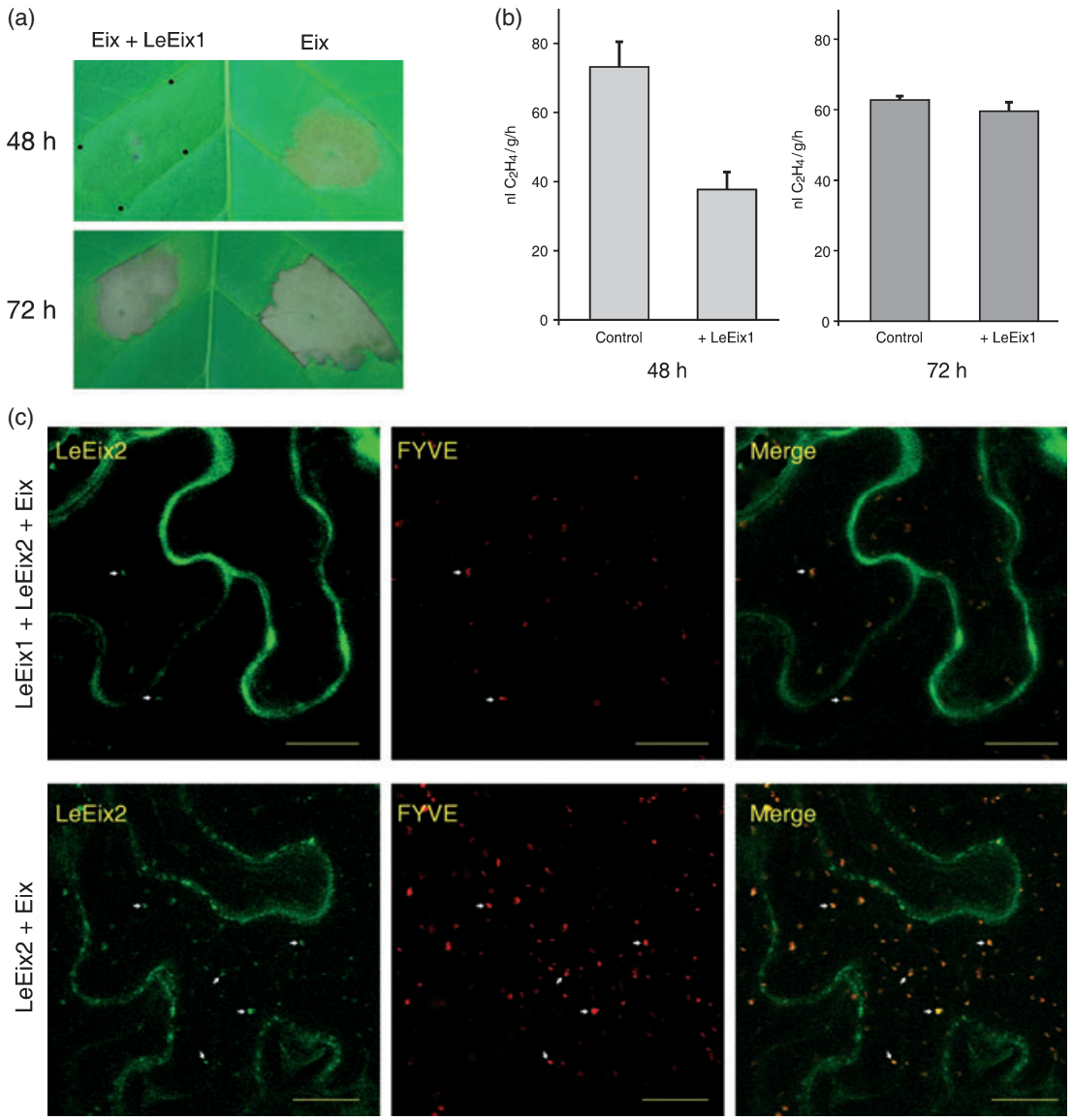

Figure 3. Hypersensitive response (HR) monitoring, ethylene production and endocytosis in tobacco plants.
(a) *Nicotiana tabacum* transiently transformed with a mixture of Pro_{35S}:*tvEix* and Pro_{35S}:*LeEix1* as indicated. The HR was monitored 48 h and 72 h after transformation.
(b) *Nicotiana tabacum* leaves transiently transformed with Pro_{35S}:*GFP* (mock) or Pro_{35S}:*LeEix1*were sampled 48 h and 72 h after transformation. Leaf disks were floated on a 250 mM sorbitol solution with 2.5 μg ml^{-1} ethylene-inducing xylanase (Eix). Ethylene biosynthesis was measured after 4 h. Values are the means and standard error (SEM) of five experiments (n = 3).
(c) *Nicotiana benthamiana* transiently transformed with a combination of Pro_{35S}:*GFP-LeEix2*, Pro_{35S}:*LeEix1* and Pro_{35S}:*FYVE–DsRed* (as indicated). Eix (2.5 μg ml^{-1}) was applied 48 after transformation. Internalization of GFP-LeEix2 on FYVE endosomes was examined 30 min after Eix application alone (bottom) or in the presence of LeEix1 (top). Arrowheads indicate co-localization of LeEix2 and FYVE on endosomes. Bar = 10 μm.

carrying the LRR domain of *LeEix2* in the bait and *BAK1* in the prey did not grow in the absence of leucine and were white when grown on X-gal plates (Figure 4). In addition, we found that BAK1 interacts with LeEix1 receptor but not the LeEix2 receptor in the BiFC system, irrespective of (i.e. with or without) Eix treatment. YC-BAK1, YN-LeEix1 and YN-LeEix2 were examined for fluorescence with the complementary half of the YFP protein and the results were negative (Figure S1).

BAK1 is required for the inhibitory activity of LeEix1 on LeEix2 signaling

The observation that BAK1 interacts with LeEix1 but not LeEix2 prompted us to examine whether BAK1 may be involved in attenuation of Eix signaling. We applied the virus-induced gene silencing (VIGS) system, using the same silencing fragments previously used (Heese *et al.*, 2007), to silence *NbBAK1* in *N. benthamiana* (Heese *et al.*, 2007), and

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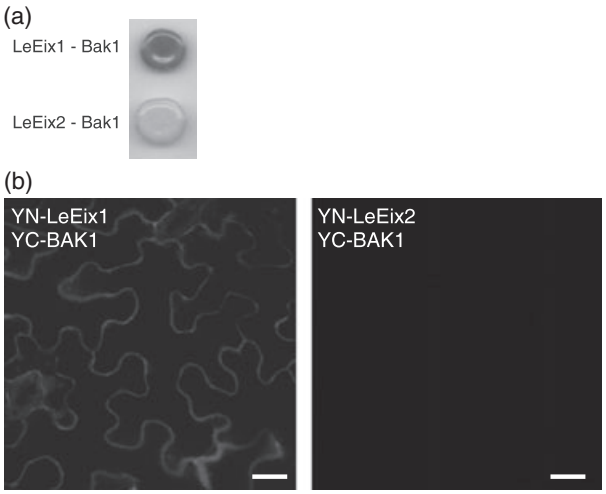

Figure 4. Interaction between LeEix1, LeEix2 and BAK1.
(a) EGY48 yeast cells containing *LeEix1* or *LeEix2* (in pEG202) and *BAK1* (in pJG4-5) were grown on galactose medium lacking the amino acids His, Ura and Trp and supplied with X-Gal.
(b) Fluorescence images of *Nicotiana benthamiana* leaves infiltrated with a mixture of *Agrobacterium* GV3101 (OD600 = 0.1) containing Pro_{35S}:*YN-LeEix1*, Pro_{35S}:*YN-LeEix2*, Pro_{35S}:*YC-BAK1* as indicated. Leaf sections were visualized 48 h after injection, under a laser-scanning confocal microscope. Bar = 20 μm.

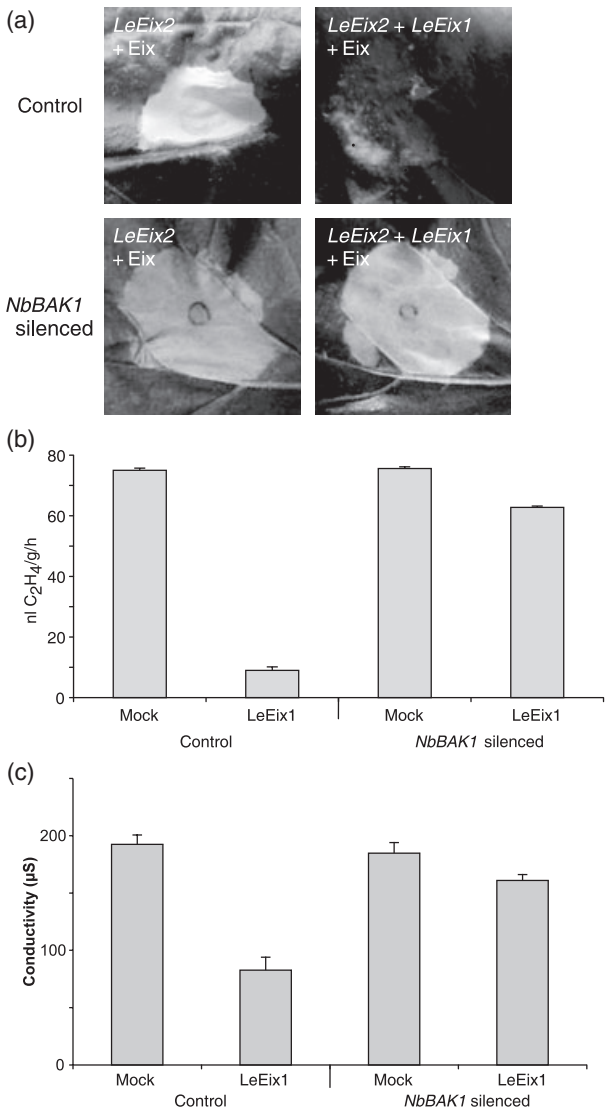

Figure 5. Hypersensitive response (HR) monitoring, ethylene production and ion leakage in *NbBAK1* silenced plants.
(a) *NbBAK1* silenced and control silenced *Nicotiana benthamiana* plants stably expressing *LeEix2* were transformed with a mixture of Pro_{35S}:*tvEix* and Pro_{35S}:*LeEix1* (as indicated). Induction of the HR was monitored 48 h after injection.
NbBAK1 silenced and control silenced *N. benthamiana* stably expressing *LeEix2* were transformed with Pro_{35S}:*LeEix1* or Pro_{35S}:*GFP* (mock). Ethylene-inducing xylanase (Eix; 2.5 μg ml^{-1}) was applied 48 h after transformation.
(b) Ethylene biosynthesis was measured after 4 h. Values are the means and SE of five experiments (n = 10).
(c) Ion leakage was measured 16 h post-Eix application. Values are the means and SE of three experiments (n = 5).

proceeded to assess the ability of *LeEix1* to attenuate Eix signaling in *NbBAK1* silenced plants as compared to control silenced plants. The silencing assays were carried out on *N. benthamiana* plants expressing *LeEix2*. The silenced plants exhibited the characteristic curled leaf phenotype described (Heese *et al.*, 2007) and had a decreased level of *NbBAK1* mRNA (Figure S4), while the expression of *NbSerk2* (Heese *et al.*, 2007; Postel *et al.*, 2010), which is related to *NbBAK1*, was not affected (Figure S4), indicating specific silencing of *NbBAK1*. As can be seen in Figure 5, silencing of *NbBAK1* impairs the ability of LeEix1 to attenuate Eix-induced responses. Inhibition of HR by LeEix1 is diminished in *NbBAK1* silenced plants compared with control plants (Figure 5a), as are both ethylene production (Figure 5b) and ion leakage (Figure 5c). In *NbBAK1* silenced plants LeEix1 inhibits about 0–15% of the Eix response, as opposed to 45–65% in the control vector silenced plants.

BAK1 is required for the inhibitory activity of LeEix1 on LeEix2 endocytosis

As we had observed that LeEix1 attenuates the endocytosis of LeEix2 on FYVE endosomes, we examined the effect of BAK1 silencing on this aspect as well. BAK1 silenced *N. benthamiana* plants were transiently transformed with a combination of Pro_{35S}:*GFP-LeEix2*, Pro_{35S}:*LeEix1* and Pro_{35S}:*FYVE–DsRed*. Eix (2.5 μg ml^{-1}) was applied 48 h after transformation. Co-localization of GFP-LeEix2 on FYVE endosomes was examined 30 min after Eix application alone or in the presence of LeEix1 (Figure 6). We examined 20 separate cells in six independent experiments. While LeEix1 expression interferes with LeEix2 endocytosis in control silenced plants, it does not interfere with LeEix2 endocytosis in *NbBAK1* silenced plants. This is particularly interesting given previous reports that endocytosis of plant defense receptors can be compromised in a *BAK1*-deficient background (Chinchilla *et al.*, 2007).

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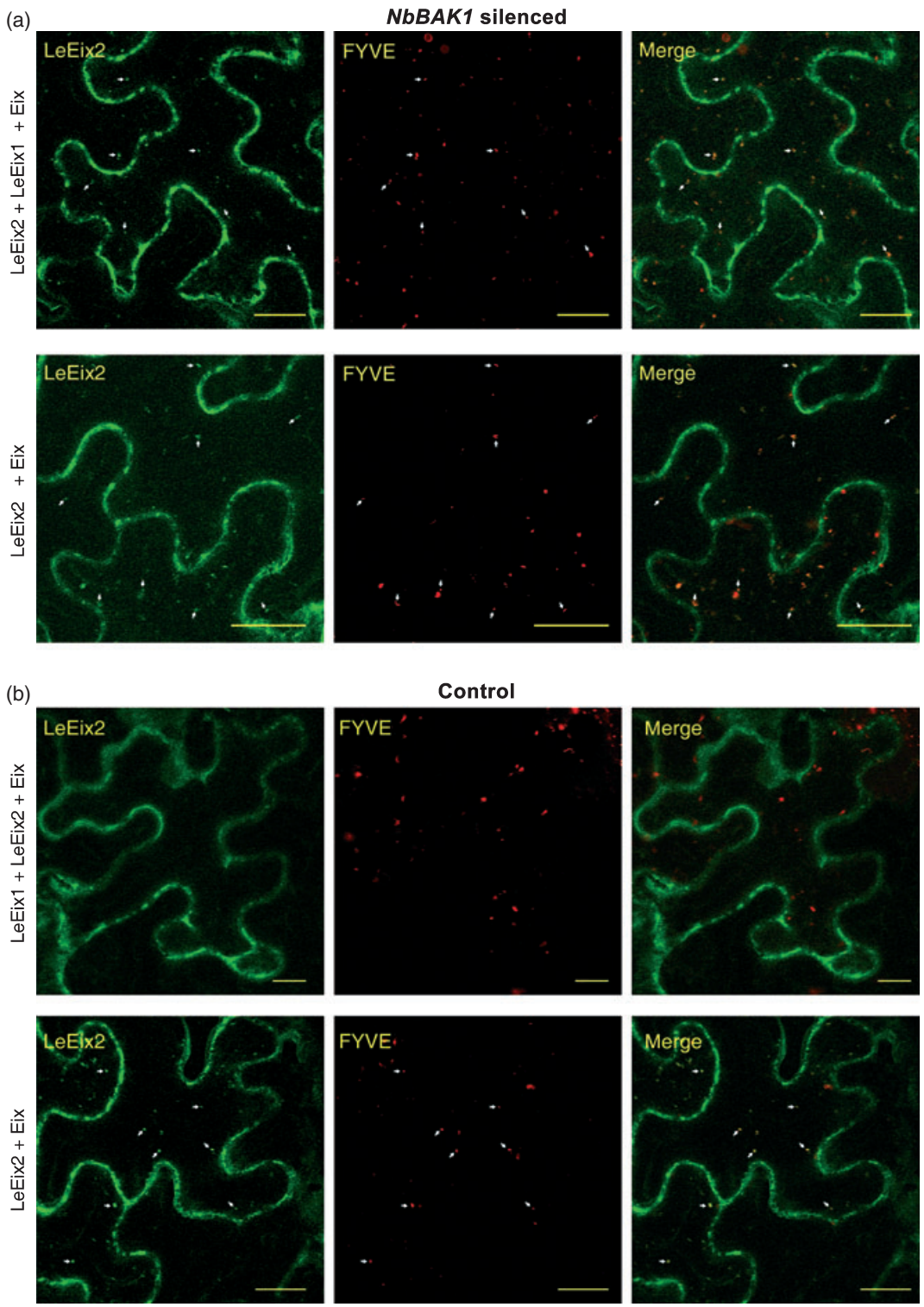

Figure 6. LeEix1 inhibits LeEix2 internalization in response to ethylene-inducing xylanase (Eix) only in the presence of BAK1.
(a) *NbBAK1* silenced and (b) control silenced *Nicotiana benthamiana* were transiently transformed with combination of Pro_{35S}:*GFP-LeEix2*, Pro_{35S}:*LeEix1* and Pro_{35S}:*FYVE–DsRed* (as indicated). Eix (2.5 μg ml^{-1}) was applied 48 h after transformation. Internalization of GFP-LeEix2 on FYVE endosomes was examined 30 min after Eix application alone or in the presence of LeEix1 (as indicated). Arrowheads indicate co-localization of LeEix2 and FYVE on endosomes. Bar = 20 μm.

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The kinase activity of BAK1 is required for BAK1-mediated attenuation of Eix-induced signaling

Our observations that BAK1 is required for attenuation of Eix-induced signaling by LeEix1 are demonstrated in Figure 5. BAK1 is known to possess kinase activity and some of its effects in plant defense responses have been linked to this activity (Chinchilla *et al.*, 2009). To examine whether BAK1 kinase activity is required for attenuation of Eix signaling we employed a BAK1 mutant deficient in its kinase activity, BAK1_K317E (AtBAK1*) (Li *et al.*, 2002). *NbBAK1* silenced *N. benthamiana* plants were transiently transformed with wild-type *AtBak1* or *AtBAK1** (Li *et al.*, 2002). The induction of the HR and ethylene biosynthesis by Eix were examined as detailed above. The expression of *AtBAK1* or *AtBAK1** in the *NbBAK1* silenced plants was examined by semi-quantitative RT-PCR using specific Arabidopsis primers that do not amplify the *N. benthamiana* gene (Figure S4). The expression level of AtBAK and AtBAK* was similar in *NbBAK1* and control silenced plants (Figure S5). Expression of wild-type *BAK1* from Arabidopsis was able to rescue the phenotype of *N. benthamiana* silenced *NbBAK1* plants, restoring the ability of LeEix1 to attenuate Eix signaling in these plants (Figure 7). However, the mutated BAK1 lacking kinase activity, BAK1_K317E (Li *et al.*, 2002), was not able to rescue the phenotype of *NbBAK1* silenced *N. benthamiana* plants (Figure 7; compare with ethylene and HR results in *NbBAK1* silenced plants in the presence of LeEix1 as detailed in Figure 5).

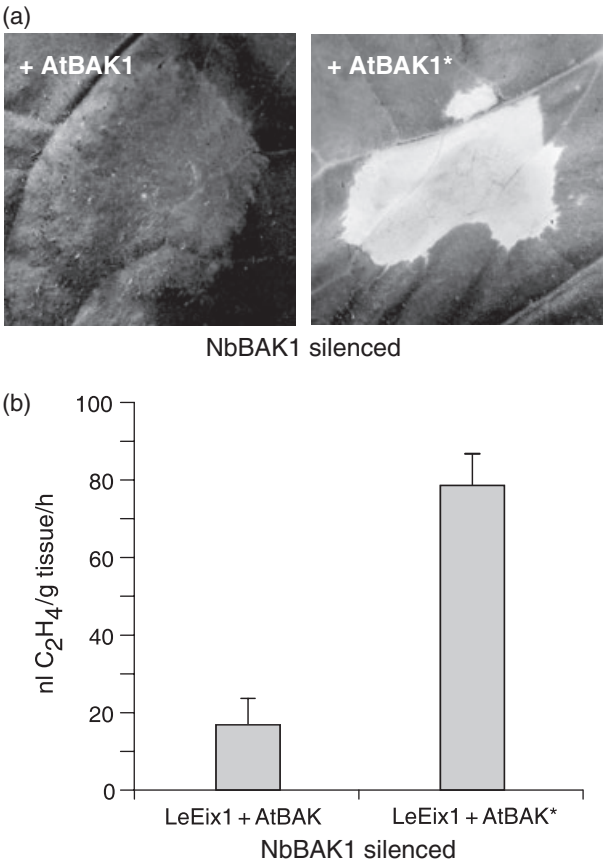

Figure 7. Kinase active BAK1 is required for the inhibitory activity of LeEix1.
NbBAK1 silenced *Nicotiana benthamiana* plants stably expressing LeEix2 and transiently expressing LeEix1, ethylene-inducing xylanase (Eix) and Arabidopsis BAK1 or LeEix1, Eix and AtBAK1_K317E (BAK1*; kinase-deficient BAK1). Induction of the hypersensitive response (HR) was monitored 48 h after injection (top panel).
Induction of ethylene biosynthesis was measured 4 h post-Eix application to *NbBAK1* silenced plants transiently expressing LeEix1 and Arabidopsis BAK1 or LeEix1 and AtBAK1_K317E (bottom panel). Values are the means and SE of three experiments (n = 6).

DISCUSSION

A dual system in which two receptors can bind a ligand but only one receptor can mediate internalization and signaling of the ligand offers many possibilities for analysis. We have discovered that LeEix1 not only fails to transmit Eix-induced signaling, but actively attenuates this signaling upon overexpression. LeEix1 could be considered a 'decoy' receptor, binding the ligand Eix with the express purpose of blocking Eix signaling. Examples of such receptors are well known in mammals (Mantovani *et al.*, 1996; Bengtsson and Ryan, 2002). One of the better characterized mammalian decoy receptor families is the DcR family of tumor necrosis factor (TNF) decoy receptors (Ashkenazi and Dixit, 1999). The decoy receptor DcR3 binds the ligands of several TNF-superfamily members, resulting in competition for ligand binding and inhibition of TNF signaling (Ashkenazi, 2002). Interestingly, several types of tumors have been found to express DcR3, thereby reducing TNF signaling, probably as a mechanism for tumor proliferation (Ashkenazi, 2002).

Our work reports on a possible decoy receptor in plants and in plant defense responses. LeEix1 may parallel the mammalian DcR TNF decoy receptors, which function in the inhibition of tumor cell death, in that it functions in the inhibition of plant defense signaling and plant cell death in response to Eix.

Similarly, LeEix1 may 'dilute' the signal in response to Eix by virtue of binding the ligand and competing with LeEix2 for Eix binding, resulting in a decrease in Eix signaling. However, it would seem that an additional mechanism is also at play, as the attenuation of LeEix2 signaling by LeEix1 is BAK1 dependent. An additional hypothesis therefore leads to the suggestion that there is a physiological function for attenuation of Eix signaling, which relies on additional mechanisms besides competition between the two receptors. We have recently reported that the endocytic protein EHD2 can inhibit LeEix2 endocytosis as well as Eix signaling upon overexpression (Bar and Avni, 2009a). It would seem that there is tight regulatory control on Eix signaling, perhaps

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one of the goals of which is to limit HR and cell death in cases where it is not warranted; this could partially account for the attenuation of Eix signaling by LeEix1 as well. As we have demonstrated, Eix application triggers *LeEix1* expression, upon which LeEix1 acts to inhibit the defense response in the short term, attenuating endocytosis, ion leakage and ethylene production in response to Eix, and attenuating the HR. Longer exposure to Eix leads to a fuller defense response including HR, suggesting that the decoy receptor function is useful particularly in the short term but does not prevent defense responses from occurring when necessary. Interestingly, LeEix1 and LeEix2 heterodimerize upon Eix application. Though we have not examined the functionality of this binding in the context of Eix-induced defense responses, it is possible that binding of LeEix1 to LeEix2 relates to the decoy activity of LeEix1.

BAK1 has been reported to be involved in the defense responses mediated by additional plant receptors (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Fradin *et al.*, 2009). BAK1 is required for proper defense receptor signaling by the FLS2 and Ve1 receptors (Chinchilla *et al.*, 2007; Heese *et al.*, 2007; Fradin *et al.*, 2009). Lack of BAK1 leads to inhibition of endocytosis and a decrease in signaling in the case of flagellin (Chinchilla *et al.*, 2007). However, we have found that silencing of *NbBAK1* may act to enhance Eix signaling, as it is crucial for attenuation of Eix signaling by LeEix1. A deficiency in BAK1 did not interfere with LeEix2 endocytosis. New functionalities are constantly emerging for BAK1. The involvement of BAK1 in the LeEix/Eix system may relate to different functionalities than its involvement in the FLS2 system, as endocytosis of LeEix2 and FLS2 occur in different pathways (Bar and Avni, 2009a). In the context of Eix-induced signaling, the requirement for BAK1 may be related to kinase activity, as we have demonstrated above that a kinase-deficient BAK1 mutant (Li *et al.*, 2002) failed to rescue the *NbBAK1* silenced phenotype and failed to restore the ability of LeEix1 to attenuate Eix-induced signaling. Based on our results, one possibility may be that BAK1 interacts with LeEix1 in the cell membrane under a certain steady state. Upon Eix application, the BAK1–LeEix1 complex binds LeEix2. This binding serves to prevent the internalization and signaling of LeEix2.

After several hours of exposure to Eix, we see a large increase in the expression of LeEix1, an *in vivo* situation which mimics LeEix1 overexpression, creating a new steady state in which LeEix1 greatly attenuates Eix signaling, as we have demonstrated herein. We suggest that LeEix1 can partially inhibit the signaling as certain Eix-induced responses such as ion leakage and ethylene production occur while LeEix1 is still at induced levels. In the absence of BAK1, the BAK1–LeEix1 complex is not present and therefore it cannot bind LeEix2 and attenuate the signaling.

Taken together our results demonstrate a key role for BAK1 and LeEix1 in fine-tuning of defense responses in plants, and open new avenues for investigating the mechanisms that control these critical processes.

EXPERIMENTAL PROCEDURES

Protein interaction assays

BiFC analysis. The *LeEix1 and LeEix2* receptors (both YN and YC) were sub-cloned in two stages: the signal peptide (*LeEix2* forward primer: 5′-tcccatgggcaaaagaactaatcc-3′; *LeEix2* reverse primer: 5′-ggccatggtccaaaagctgtctctagg-3′; *LeEix1* forward primer: 5′-gccatgg acaaatggaaatatgcaagattag-3′; *LeEix1* reverse primer: 5′-ggccatggaccacctaatccaaaag-3′) was cloned into the *Nco*I site upstream of the YFP gene fragment and the rest of the genes (*LeEix2* forward primer: 5′-gggtcgacattaacttcaagagaag-3′; *LeEix2* reverse primer: 5′-ctggtcgactcagttccttagctttccc-3′; *LeEix1* forward primer: 5′-gggtcgactaacaagaccctatg-3′; *LeEix1* reverse primer: 5′-cagtcgaccattcatcgggccctcctcagc-3′) was cloned into the *Sal*I site of both pSY751 and pSY752 (Bracha-Drori *et al.*, 2004), downstream of the YFP gene fragment. *BAK1* (forward primer: 5′-catgggatccggaacgaagattaatgatc-3′; reverse primer: 5′-catgggatcc ttactcttggacccgaggg-3′) was sub-cloned into the *Bam*HI site of pSY752. The resulting plasmids, pSY751-LeEix2 (YN-LeEix2), pSY751-LeEix1 (YN-LeEix1) pSY752-LeEix2 (YC-LeEix2), pSY752-LeEix1 (YC-LeEix1) and pSY752-BAK1 (YC-BAK1) were used for transient expression assays in *N. benthamiana*.

Yeast two-hybrid analysis. Two-hybrid analyses were performed essentially as in Gyuris *et al.* (1993). The LRR domains of LeEix1 or LeEix2 were cloned in the 'bait' plasmid pEG202 using the following primers: *LeEix1-LRR* forward primer: 5′-gggcggccgcctctgcttgagttggagtacttgaattacttg-3′; *LeEix1-LRR* reverse primer: 5′-gggcggccgctcacagttgagtgcttgatgg-3′; *LeEix2-LRR* forward primer: 5′-gggcggccgcctctacttgagttggagtatttgaa-3′; *LeEix2-LRR* reverse primer: 5′-gggcggccgctcacagttgagtgcttgatgg-3′ and in the 'prey' plasmid pJG4-5 *LeEix1-LRR* forward primer: 5′-ctggaattcctgcttgagttggagtacttgaattacttg-3′; *LeEix1-LRR* reverse primer: 5′-gccctcgagtcacagttgagtgcttgatgg-3′; *LeEix2-LRR* forward primer: 5′-ctggaattcttgcgccttggtggtaacg-3′; *LeEix2-LRR* reverse primer: 5′-gccgaattctcatcctgataagtggttgttc-3′. AtBAK1 was obtained from Dr Russinova (Ghent University, Belgium) and Dr de Vries (Wageningen University, The Netherlands). BAK1 was cloned into the *Eco*RI–*Xho*I sites in pJG4-5 using the primers catggaattcacgaagattaatgatc and catgctcgagttactcttggacccgaggg.

The constructs were transformed into the yeast strain EGY48 to test for protein interaction.

Construction of plasmids and protein expression. FYVE-DsRed was obtained from Dr Jozef Samaj (Institute of Cellular and Molecular Botany, University of Bonn). VIGS silencing vectors were obtained from Dr Dinesh-Kumar (Plant Biology, University of California, Davis) and Dr Rahtjen (The Sainsbury Laboratory, John Innes Centre, Norwich). LeEix1 and LeEix2 are as described in Ron and Avni (2004). GFP-LeEix, as described in Bar and Avni (2009a).

AtBAK1 was obtained from Dr Russinova (Ghent University, Belgium) and Dr de Vries (Wageningen University, The Netherlands) (Russinovaa *et al.*, 2004). AtBAK1 was cloned upstream of the HA tag into the *Nco*I site of the pPILY vector (Ferrando *et al.*, 2000); the entire cassette was then sub-cloned into the pART27 (Gleave, 1992), generating Pro_{35S}:*BAK1-HA.*

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Transient expression was performed as previously described (Ron and Avni, 2004). Protein expression in leaves was observed 24–72 h after injection.

Elicitor application. Eix was purified from Sigma crude xylanase extract (http://www.sigmaaldrich.com) as previously reported (Dean *et al.*, 1991) and applied to cell cultures or leaf disks at a final concentration of 2.5 $\mu g/ml^{-1}$ or applied to the petiole of detached leaves at a final concentration of 2.5 $\mu g\ g^{-1}$.

Microscopy. Cells were analyzed using a Zeiss LSM510 or Meta confocal laser scanning microscope (Zeiss, http://www.zeiss.com/) with the following configuration: 30 mW argon and HeNe lasers, 458, 488, 514 or 458, 477, 488, 514 and 541 or 568 maximum lines respectively. All images depict single sections. Contrast and intensity for each image were manipulated uniformly using Adobe Photoshop (http://www.adobe.com/) and/or ImageJ software (http://rsbweb.nih.gov/ij/).

Ethylene and ion leakage measurements. Ethylene biosynthesis was measured in a gas chromatograph 4 h post-Eix application as previously described (Bar and Avni, 2009a).

Ion leakage was measured 18 h post-Eix application. Six leaf disks (1 cm diameter) of *N. tabacum* cv. *Samsun* were sampled from treated areas for each plant floated in 15 ml double-distilled water (DDW) with gentle shaking. Conductivity was measured with an autoranging EC/Temp Meter TH-2400 (EI-Hamma Instruments, http://www.elhamma.com).

RNA analysis. Leaves were ground with a mortar and pestle and 100–200 mg of powder was used for total RNA isolation using the SV total RNA isolation kit (Promega, http://www.promega.com/) according to manufacturer's protocol.

Tomato plants were infiltrated with a solution of 2 $\mu g\ ml^{-1}$ Eix or a control solution. Total RNA was extracted from leaf samples collected at indicated time points. RNA from each time point was subjected to northern blot analysis with either a common LeEix probe, or a 3′ untranslated region (UTR)-specific LeEix1 or LeEix2 probe.

RT-PCR analysis. Total RNA was extracted from leaves of 4–6-week-old *N. benthamiana* silenced plants or detached *S. lycopersicum* cv. M82 leaves following elicitor treatments as indicated using the SV total RNA isolation kit (Promega) according to the manufacturer's instructions. RNA (0.5–2 μg) was converted to cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). Two microliters of each reverse transcriptase reaction were used as a template in a PCR reaction containing the following specific primer pairs: LeEix1/2 FOR: 5′-ccaggagtccgagtacaagaatgc-3′; LeEix1 REV: 5′-cagcggccgccattcatcgggccctcctcagc-3′; LeEix2 REV: 5′-ctggcggccgctcagttccttagctttccc-3′; GAPDH FOR: 5′-atgctcccatgtttgttgtgggtg-3′; GAPDH REV: 5′-ttagccaaaggtgcaaggcagttc-3′. NbBAK1 FOR: 5′-tcctgacggaccatctcctcttt-3′; NbBAK1 REV: 5′-gctcataactgggcaaagggctt-3′. Tobacco Actin FOR: 5′-tggaaagattgtactcagtggcg-3′; Tobacco Actin REV: 5′-agaagcattttctgtgcacaatgg-3′. AtBAK1 FOR 5′-ggttgttaaaagagaagaaattg-3′ AtBAK1 REV 5′-ccgaggggtattcgttttcgatc-3′. Quantification of the resultant PCR reactions was performed using ImageJ software.

Virus-induced gene silencing assay. Virus-induced gene silencing assays were performed as described by Liu *et al.* (2002). pTRV1, pTRV2 and pTRV-*tPDS* VIGS vectors were obtained from Dr Dinesh-Kumar. Two independent BAK1 silencing vectors were obtained from Dr Rathjen. Five weeks after tobacco rattle virus (TRV) infection, silenced plants were challenged with Pro_{35S}:*tvEix* (Ron and Avni, 2004).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Bimolecular fluorescence complementation analysis of the interaction between LeEix1 LeEix2, BAK1 and control proteins.

Figure S2. Expression of LeEix1, LeEix2 and BAK1 in yeast cells.

Figure S3. Induction of LeEix transcripts in response to ethylene-inducing xylanase (Eix).

Figure S4. Expression of *NbBAK1* and *NbSerk2* in control and *NbBAK1* silenced plants.

Figure S5. Expression of AtBAK1 and AtBAK* in control and NbBAK1 silenced plants.

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