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# EHD2 inhibits ligand-induced endocytosis and signaling of the leucine-rich repeat receptor-like protein LeEix2

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## SUMMARY

**Plants are constantly being challenged by aspiring pathogens. In order to protect themselves, plants have developed numerous defense mechanisms that are either specific or non-specific to the pathogen. Pattern recognition receptors can trigger plant defense responses in response to specific ligands or patterns. EIX (ethylene-inducing xylanase) triggers a defense response via the LeEix2 receptor, while bacterial flagellin triggers plant innate immunity via the FLS2 receptor. Endocytosis has been suggested to be crucial for the process in both cases. Here we show that the EIX elicitor triggers internalization of the LeEix2 receptor. Treatment with endocytosis, actin or microtubule inhibitors greatly reduced the internalization of LeEix2. Additionally, we demonstrate that plant EHD2 binds to LeEix2 and is an important factor in its internalization and in regulation of the induction of defense responses such as the hypersensitive response, ethylene biosynthesis and induction of pathogenesis-related protein expression in the case of EIX/LeEix2 (an LRR receptor lacking a kinase domain), but does not appear to be involved in the FLS2 system (an LRR receptor possessing a kinase domain). Our results suggest that various endocytosis pathways are involved in the induction of plant defense responses.**

**Keywords: LRR-RLP, LeEix, EIX, EH domain, endocytosis, FLS2.**

## INTRODUCTION

In recent years, a significant body of evidence has accumulated suggesting a role for regulated endocytosis in plant development and plant immunity (Robatzek, 2007). A broad range of molecular markers have been developed, and, together with lipid marker dyes, are used to analyze plasma membrane vesicular recycling and endocytosis as well as to identify and characterize the corresponding endomembrane compartments in plant cells (Samaj *et al.*, 2004, 2005; Gross *et al.*, 2005; Lam *et al.*, 2007; Muller *et al.*, 2007). The role of endocytosis in plant immunity and plant defense responses requires further elucidation.

Elicitors (microbe-associated molecular patterns (MAMPs)) that trigger plant defense responses have been isolated from a variety of phytopathogenic and non-pathogenic micro-organisms (Fuchs *et al.*, 1989; Ricci *et al.*, 1993; Ebel and Cosio, 1994; Felix *et al.*, 1999). Elicitors have been shown to enter plant cells in several cases. In soybean cell cultures, the *Verticillium* elicitor was shown to enter the cell by an endocytic process (Horn *et al.*, 1989). Flg22 was shown to stimulate endocytosis of FLS2 in a process that requires kinase activity (Robatzek *et al.*, 2006). Recently, the cryptogein elicitor was shown to induce endocytosis that correlated with its activation of the defense response (Leborgne-Castel *et al.*, 2008).

The fungal protein elicitor EIX (ethylene-inducing xylanase), induces ethylene biosynthesis, electrolyte leakage, expression of 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). EIX was shown to specifically bind to the plasma membrane of responsive cultivars of both tomato and tobacco (Hanania and Avni, 1997). The response to EIX in tobacco and tomato cultivars is controlled by a leucine-rich repeat receptor-like protein (LRR-RLP) encoded by a single dominant locus, termed LeEix (Ron and Avni, 2004).

Leucine-rich repeat receptor kinases (LRR-RLKs) and LRR-RLPs have been implicated in signaling as well as defense responses in plants (Becraft, 2002). The most intensively studied LRR-RLK in the context of plant defense responses is 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). FLS2 recognition of flg22 leads to a response that includes generation of reactive oxygen species (ROS), MAP kinase 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).

In the case of LRR-RLPs, although less information is available, they have also been implicated in responses to pathogens. The tomato *Cf* genes that mediate resistance to *Cladosporium fulvum* encode LRR-RLPs, the LRR domain of which was shown to be important for avirulence (*Avr*) gene recognition (Takken *et al.*, 1999; van der Hoorn *et al.*, 2005). Additional LRR-RLPs include the tomato Ve resistant proteins (Kawchuk *et al.*, 2001) and the LeEix proteins, as mentioned above (Ron and Avni, 2004).

Interestingly, while the tomato Ve2, Cf9, Cf4 and LeEix resistant proteins (Jones *et al.*, 1994; Takken *et al.*, 1998; Kawchuk *et al.*, 2001; Ron and Avni, 2004) contain the conserved endocytosis signal Yxx$\phi$ within the short cytoplasmic domain, FLS2 does not contain a Yxx$\phi$ motif, but has been reported to contain a PEST-like motif that has also been implicated in endocytosis (Robatzek *et al.*, 2006). A mutation in the endocytosis motif of LeEix2 resulted in abolishment of HR induction in response to EIX, suggesting that endocytosis plays a key role in mediating the signal generated by EIX (Ron and Avni, 2004). Similarly, it has been reported that impairing the PEST-like motif in FLS2 may compromise FLS2 internalization and abolish some elements of the flg22-triggered defense response (Robatzek *et al.*, 2006).

Receptor-mediated endocytosis has also been reported to be important for the response to pathogens in mammalian systems, such as in the case of the Toll-like receptors, which also contain extracellular LRR domains (Husebye *et al.*, 2006). Study of mammalian systems has also shown that endocytosis depends on a large number of protein–protein interactions mediated by specific modules. One such module is the EH (Eps15 homology) domain, first identified in Eps15 (Wong *et al.*, 1995; Carbone *et al.*, 1997). A recent study (Bar *et al.*, 2008) demonstrated that proteins containing the EH domain are also involved in endocytosis in plants.

In this work, we show that EIX triggers internalization of the LeEix2 receptor on endosomes, which is dependent on an intact cytoskeleton. Additionally, we demonstrate that plant EHD2 (Bar *et al.*, 2008) is an important factor in the internalization and downstream signaling of EIX/LeEix2, but does not appear to be involved in FLS2 signaling.

## RESULTS

### Ligand-induced endocytosis of LeEix2 in *Nicotiana tabacum* cells

EIX has previously been shown to enter EIX-responsive *N. tabacum* cells and protoplasts (Hanania *et al.*, 1999; Rotblat *et al.*, 2002). It was also demonstrated that mutation in the endocytosis signal of the EIX receptor LeEix2 caused the HR in response to EIX treatment to be abolished (Ron and Avni, 2004). In view of this, we wished to determine whether the LeEix2 receptor enters the cell as a result of EIX treatment.

A GFP-tagged LeEix2 protein transiently expressed under the viral 35S promoter in non-EIX-responsive cultivars retained its ability to induce a HR upon EIX treatment in a manner similar to the wild-type receptor (Figure S1). Although the 35S promoter is usually very strong, expression of GFP–LeEix2 was much lower than is typical for this promoter. GFP–LeEix2 was localized to the plasma membrane in transiently transformed *Nicotiana benthamiana* cells (Figure 1a). Detached leaves of *N. benthamiana* transiently transformed with *Pro$_{35S}$:GFP-LeEix2* were treated with EIX (2.5 $\mu$g g$^{-1}$ tissue) by petiole application. The GFP-tagged LeEix2 receptor was visible on FYVE-positive endosomes 10–15 min after EIX application (Figure 1b). GFP-tagged LeEix2 did not co-localize with the FYVE marker in untreated leaves (Figure 1a). The FYVE domain has been reported to localize to endosomes in mammals (Stenmark *et al.*, 1996) as well as plants (Jensen *et al.*, 2001; Heras and Drobak, 2002; Voigt *et al.*, 2005). The FYVE-positive LeEix2-positive were highly motile (Movie S1); in untreated leaves, the FYVE-positive endosomes have similar motility, but GFP–LeEix2 is localized to the plasma membrane (Movie S2). LeEIX2 generally disappeared from endosomes (or the amount of LeEIX2 was greatly reduced) about 60–90 min after EIX application (Figure S2). GFP–LeEix2 started to appear on endosomes 10–15 min after petiole application of EIX in cells in the vicinity of the leaf central vein. The maximum number of GFP–LeEix2-stained endosomes was observed at about 25–35 min after EIX application, and the number of GFP–LeEix2-stained endosomes declined dramatically 60–90 min after application (Figure S2). Throughout the experiment, GFP–LeEix2 also remained localized to the plasma membrane, probably due to over-expression of the protein (the amount of receptor required to internalize the applied ligand is probably not microscopically visible compared with the total amount of receptor in the cell). Internalization following a similar time course was observed in cells closer to the periphery of the leaf, but the internalization started later in such cases, as it takes the EIX longer to reach the intracellular space surrounding these cells. Images obtained from a single cell demonstrate that the number of GFP–LeEix2/FYVE co-localized pixels declines between 25 and 45 min after EIX application (Figure S3). Interestingly, addition of the protein synthesis inhibitor cycloheximide (CHX; 50 $\mu$M) did not affect the internalization, but caused GFP–LeEix2 to remain on the endosomes for greater periods of time. Figure S2 shows that, although GFP–LeEix2 disappears from endosomes 60–90 min after EIX application, the receptor remains on endosomes 120–180 min after

 

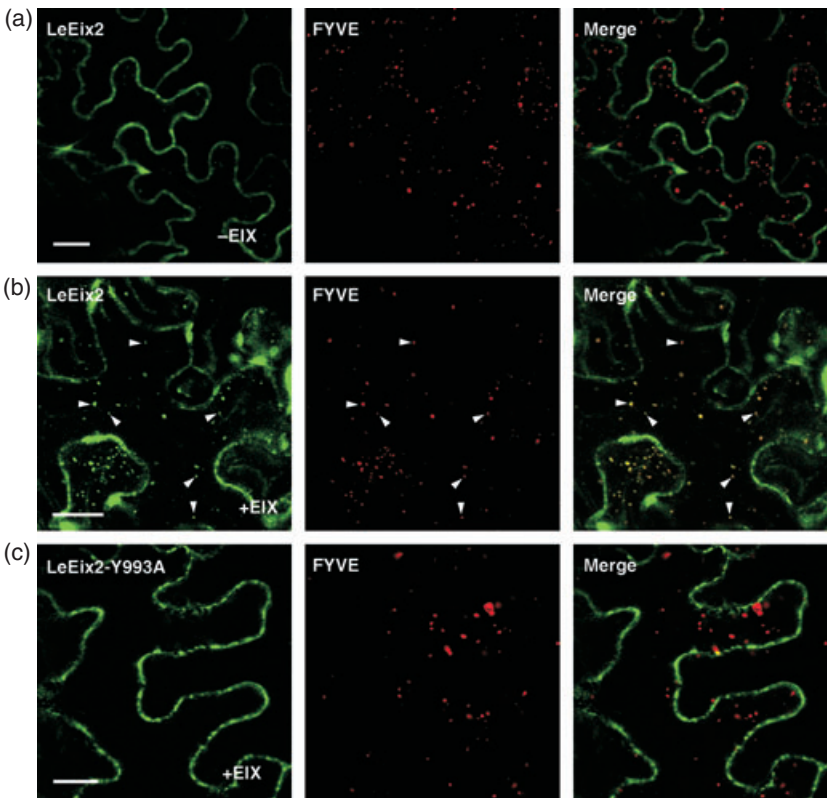


**Figure 1.** Localization of LeEix2 with the endosomal marker FYVE–DsRed in leaf tissue.
(a,b) *N. benthamiana* transiently expressing GFP–LeEix2 and FYVE–DsRed was treated with (a) $H_2O$ or (b) EIX (2.5 μg $g^{-1}$ tissue).
(c) *N. benthamiana* transiently expressing GFP–LeEix2-Y993A and FYVE–DsRed was treated with EIX (2.5 μg $g^{-1}$ tissue).
Leaf sections were visualized under a laser-scanning confocal microscope 15–30 min after treatment. Scale bar = 20 μm. Arrowheads indicate co-localized pixels.

application in the presence of CHX. This could indicate that protein synthesis is required for GFP–LeEix2 recycling and/or degradation.

Previously, we showed that mutating the Yxxϕ domain in LeEix2 by replacing the tyrosine at position 993 with alanine, thereby generating LeEix2-Y993A, abolished its function (Ron and Avni, 2004). Detached leaves of *N. benthamiana* transiently transformed with GFP–LeEix2-Y993A were treated with EIX (2.5 μg $g^{-1}$ tissue) by petiole application. Twenty minutes after EIX application, the mutated receptor can be seen only on the plasma membrane (Figure 1c). A similar time course was used as in the experiment with the wild-type receptor, and it was found that the mutated receptor does not internalize in response to EIX application. To further characterize the phenomenon of EIX-induced LeEix2 internalization, we tested a series of inhibitors. We compared the localization of GFP–LeEix2 and FYVE–DsRed on endosomes after EIX application (Figure 1b) with their localization after treatment with EIX and various inhibitors (Figure 2). Treatment of detached leaves with wortmannin (33 μM) or LY294002 (20 μM) 20 min before EIX application (2.5 μg $g^{-1}$ tissue by petiole application) abolished staining or greatly reduced the number of endosomes stained with FYVE as well as LeEix2-positive endosomes (Figure 2a,b). Wortmannin is considered to be a specific inhibitor of phosphoinositide-3-kinases, but it has been shown to inhibit additional associated proteins (Emans *et al.*, 2002) such as phosphoinositide-4-kinase. LY294002 is a selective phosphatidylinositol-3-kinase (PI3K) inhibitor (Vlahos *et al.*, 1994), and is considered a more 'narrow' endocytosis inhibitor than wortmannin. In LY294002-treated cells, an endosome positive for both FYVE and LeEix2 occasionally remained (Figure 2b), but its motility was abolished. Wortmannin completely abolished FYVE labeling of endosomes within the cell. Treatment with 20 μM latrunculin B (which prevents actin polymerization) or 20 μM oryzalin (which prevents microtubule polymerization in plants and was shown to inhibit endosomal movement) (Baluska *et al.*, 2002, 2004; Voigt *et al.*, 2005; Dhonukshe *et al.*, 2006) also completely abolished the endosomal localization of LeEix2 (Figure 2c), indicating that actin and tubulin are required for EIX internalization. All confocal image selections of cells treated with inhibitors were captured 20–30 min after EIX application.

### AtEHD2 interacts with LeEix2

EH domain-containing proteins (EHDs) function as regulators of endocytosis through their ability to interact with other proteins (Rotem-Yehudar *et al.*, 2001). We have recently described the isolation and characterization of two Arabidopsis EH domain-containing proteins (AtEHD1 and AtEHD2), which were shown to be involved in endocytosis in plant systems. Over-expression of AtEHD2 was shown to inhibit endocytosis of FM-4-64 in plant cells and transferrin in mammalian cells (Bar *et al.*, 2008). EH domain-containing proteins have been shown to occur in complex with endo-

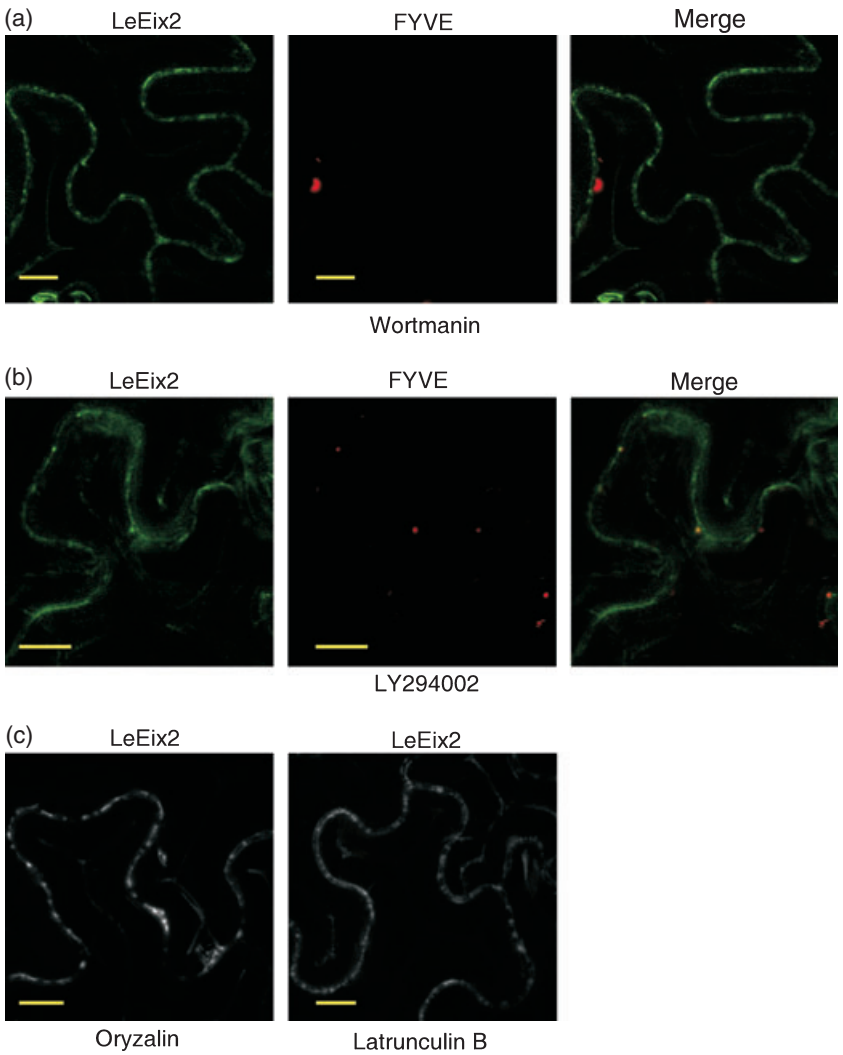


**Figure 2.** Inhibition of EIX-mediated LeEix2 internalization by various inhibitors.
(a,b) Detached leaves of *N. benthamiana* transiently expressing GFP–LeEix2 and FYVE–DsRed were incubated with (a) wortmannin (33 μM) or (b) LY294002 (20 μM).
(c,d) Detached leaves of *N. benthamiana* transiently expressing GFP–LeEix2 were incubated with (c) oryzalin (20 μM) or lantrunculin B (20 μM). All inhibitors were applied 20 min before EIX application (2.5 μg $g^{-1}$ tissue). Leaf sections were visualized under a laser-scanning confocal microscope 15–30 min after EIX application. Scale bar = 10 μm.

cytic proteins, including receptors, in mammalian systems (Lin *et al.*, 2001; Rotem-Yehudar *et al.*, 2001; Wendland, 2001; Galperin *et al.*, 2002; Guilherme *et al.*, 2004). We therefore examined whether AtEHD2 interacts with LeEix2. The cytoplasmic domain of the LeEix2 receptor seemed to be most likely to interact with EHD2 (given that EHD2 is not an integral membrane protein) and was initially examined.

Figure 3 shows that AtEHD2 interacts with the cytoplasmic domain of LeEix2 *in planta* in the BiFC system, in which active YFP is reconstituted only when non-fluorescent N-terminal (YN) and non-fluorescent C-terminal (YC) YFP fragments are brought together by protein–protein (AtEHD2–LeEix2) interactions (Bracha-Drori *et al.*, 2004). Reconstitution of YFP fluorescence was examined for transient co-expression of AtEHD2 and the cytoplasmic domain of LeEix2 (LeEix2_CD). Cells co-expressing YN–LeEix2_CD and YC–AtEHD2 showed clear YFP fluorescence localized to the cell membrane (Figure 3). YN–LeEix2_CD and YC–AtEHD2 were individually examined for fluorescence with the complementary half of the YFP protein and the results were negative (Figure 3).

To further examine the interaction between LeEix2 and EHD2, we tested the interaction between the full-length wild-type LeEix2 receptor or the full-length LeEix2-Y993A mutant receptor and EHD2, in the presence or absence of EIX. Reconstitution of YFP fluorescence was examined after treatment with EIX by petiole application to detached leaves (Figure 3). Cells co-expressing YN–LeEix2 and YC–AtEHD2 showed YFP fluorescence localized to the cell membrane only after EIX treatment (Figure 3). This could indicate that EHD2 interacts with LeEix2 only as a result of EIX treatment, or could be due to spatial separation prior to EIX treatment, as the N-terminal half of the YFP protein conjugated to the LeEix2 receptor is extracellular, while the C-terminal half of the YFP protein conjugated to EHD2 is intracellular; this spatial separation could prevent reconstitution of the YFP signal despite binding. In such a case, upon EIX treatment, the LeEix2 receptor enters the cell (as seen above) and the

 


**Figure 3.** BiFC visualization of the interaction between LeEix2 and AtEHD2.
Fluorescence images of *N. benthamiana* leaves infiltrated with a mixture of *Agrobacterium* ($OD_{600}$ = 0.1) containing *Pro*$_{35S}$*:YN-LeEix2_CD*, *Pro*$_{35S}$*:YN-LeEix2*, *Pro*$_{35S}$*:YN-LeEix2-Y993A*, *Pro*$_{35S}$*:YC-AtEHD2*, *Pro*$_{35S}$*:YN* and *Pro*$_{35S}$*:YC* as indicated.
Leaf sections were visualized 48 h after injection, with or without the application of EIX (2.5 μg $g^{-1}$ tissue), under a laser-scanning confocal microscope. Scale bar = 50 μm.

YFP signal then appears. The internalized receptor is probably localized on the inner side of the endosomal membrane. However, there is probably a phase during internalization of LeEix2 in which the YFP halves carried on EHD2 and LeEIX are in close enough proximity to reconstitute the YFP signal. Once the receptor reaches the endosome (as seen in Figure 1), it no longer interacts with EHD2, as no reconstituted YFP signal was observed on endosomes. Moreover, no interaction was observed between the mutated LeEix2-Y993A and EHD2 upon treatment with EIX (Figure 3). YN–LeEix2, YN–LeEix2-Y993A and YC–AtEHD2 were individually examined for fluorescence with the complementary half of the YFP protein and the results were negative (Figure 3).

### AtEHD2 inhibits EIX internalization

To examine the role of AtEHD2 in internalization of the LeEix2 receptor, LeEix2 and AtEHD2 were co-expressed in *N. benthamiana*. EIX was applied to detached leaves (by petiole application) 48 h after transformation, and leaf sections were visualized 15–30 min after EIX application. Figure 4(a) shows that GFP–LeEix2 vesicles were abolished upon over-expression of AtEHD2.

This phenomenon was also examined in a cell culture of an EIX-responsive cultivar of *N. tabacum* over-expressing AtEHD2, in which the degree of AtEHD2 expression is variable. The cell culture was incubated with EIX (2.5 μg $ml^{-1}$) and FM-4-64 (5 μM) for 30 min, and subsequently stained with Hoechst stain (bisbenzimide) to examine cell vitality (Elbaz *et al.*, 2002). We have previously demonstrated that EIX-induced programmed cell death is a process that commences in cell cultures of responsive cultivars as early as 30 min after inhibitor application, and that the first indication of this process is the cells becoming penetrable to Hoechst stain, which stains the nucleus, despite the fact that the cells still exhibit normal cellular morphology at this stage (Elbaz *et al.*, 2002). Inhibition of FM-4-64 entry in the presence of AtEHD2–GFP has also been demonstrated (Bar *et al.*, 2008). As can be seen in Figure 4(b), AtEHD2 inhibits both FM-4-64 internalization and programmed cell death as determined by Hoechst staining, in an expression level-dependent manner. Inhibition of EIX-dependent programmed cell death is most likely due to inhibition of LeEix2 internalization.

### AtEHD2 inhibits EIX signaling

Inhibition of EIX-induced cell death by AtEHD2 suggests that AtEHD2 not only inhibits internalization but also inhibits EIX signaling. To investigate this further, we examined the effect of AtEHD2 over-expression on the induction of an HR and ethylene biosynthesis by EIX. *Pro*$_{35S}$*:tvEix* was transiently expressed with *Pro*$_{35S}$*:AtEHD2*, *Pro*$_{35S}$*:AtEHD2-2* (Bar *et al.*, 2008), *Pro*$_{35S}$*:AtEHD1* [*AtEHD1*

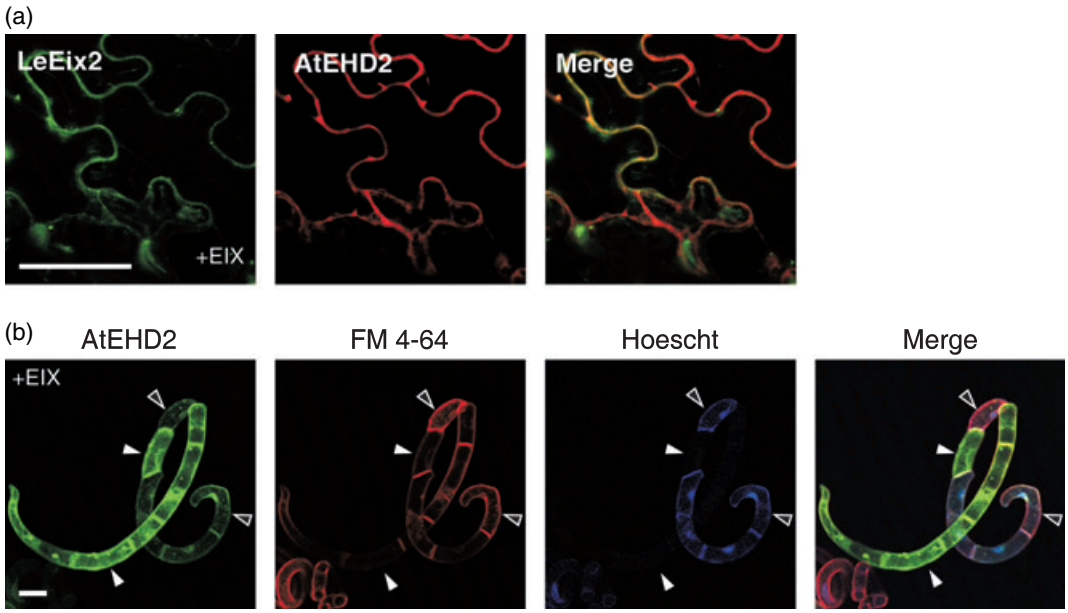


**Figure 4.** EHD2 over-expression inhibits internalization of LeEix2 and EIX.
(a) *N. benthamiana* transiently expressing GFP–LeEix2 and AtEHD2–cherry was treated with EIX (2.5 μg $g^{-1}$ tissue) and visualized 48 h after transformation under a laser-scanning confocal microscope.
(b) Tobacco cell culture of an EIX-responsive cultivar expressing AtEHD2–GFP (note varying expression levels) stained with the vitality marker Hoechst stain (1 μg $ml^{-1}$) and the endocytic tracer FM-4-64 (5 μM), 30 min after EIX treatment (2.5 μg $ml^{-1}$).
Closed arrowheads indicate cells expressing AtEHD2–GFP at a high level; open arrowheads indicate cells expressing AtEHD2–GFP at a low level.
Scale bar = 50 μm.

is sequentially and structurally similar to *AtEHD2* but was found not to inhibit endocytosis upon over-expression (Bar *et al.*, 2008)] or $Pro_{35S}$*:GFP* (mock treatment). Induction of an HR by EIX was monitored 24–96 h after injection. Leaves transiently expressing a mixture of $Pro_{35S}$*:tvEix* and $Pro_{35S}$*:AtEHD2* or $Pro_{35S}$*:AtEHD2-2* showed no HR (Figure 5a), while leaves transiently expressed a mixture of $Pro_{35S}$*:tvEIX* with either $Pro_{35S}$*:AtEHD1* or $Pro_{35S}$*:GFP* developed an HR within 48 h (Figure 5a). The inhibition of HR induction was usually complete, although occasionally an HR did occur in the AtEHD2 over-expression sample much later and only on part of the injected surface. To assess the effect of AtEHD2 on ethylene biosynthesis, *N. tabacum* leaves were transiently transformed with $Pro_{35S}$*:AtEHD2* or $Pro_{35S}$*:GFP*. Leaf discs were prepared from the transformed leaves 48 h after transformation and incubated with 2.5 μg $ml^{-1}$ EIX. Ethylene production was measured after 4 h of incubation. Figure 5(b) shows that AtEHD2 greatly reduces the amount of ethylene produced in response to EIX, but does not abolish it completely. This could be due to a minimal amount of EIX entering the cell, which may be sufficient to induce some ethylene biosynthesis, or due to partial EIX signaling as a result of binding of EIX to the LeEix2 receptor on the plasma membrane, despite the fact that the receptor cannot enter the cell. Different expression levels of AtEHD2 could also affect this result.

### Silencing EHD2 expression enhances EIX signaling to a level above wild-type

Given that EHD2 over-expression inhibits EIX signaling, we wished to examine whether knock-down of EHD2 could enhance EIX signaling. A partial EST (BP133939) from the C-terminus of *N. tabacum* EHD2 was identified in the TIGR databases. The EST, which translates into a protein fragment with 87% homology to AtEHD2, was isolated from a tobacco total leaf cDNA template and cloned into the pTRV2 vector (Liu *et al.*, 2002a) to generate pTRV2-*NtEHD2*. *Virus-induced gene silencing* assays were performed using pTRV2-*NtEHD2* as described previously (Liu *et al.*, 2002a). Five weeks after tobacco rattle virus (TRV) infection, silenced plants were challenged with $Pro_{35S}$*:tvEIX* (Ron and Avni, 2004). The induction of an HR in pTRV2-*NtEHD2*-silenced plants appeared significantly earlier compared to induction of an HR in plants treated with empty vector (Figure 6a). NtEHD2-silenced plants exhibited an HR 18–24 h after injection, when control plants had not yet developed an HR. The control plants developed an HR 48–96 h post-injection (Figure 6a). Furthermore, the induction of ethylene biosynthesis in EHD2-silenced plants was significantly higher than in control plants (Figure 6b). Semi-quantitative RT-PCR experiments indicated that the level of *EHD2* mRNA compared with the level of GAPDH mRNA in the silenced plants was reduced to about 35% of that in wild-type plants (Figure 6c). The results

 

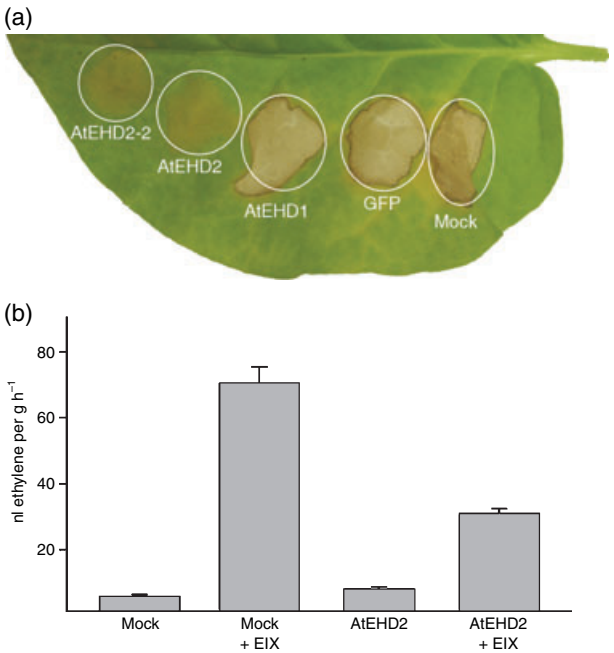


**Figure 5.** EHD2 over-expression inhibits the EIX-induced HR and ethylene biosynthesis.
(a) *N. tabacum* transiently transformed with a mixture of *Pro$_{35S}$:tvEix* and either *Pro$_{35S}$:AtEHD2*, *Pro$_{35S}$:AtEHD2-2*, *Pro$_{35S}$:AtEHD1* or *Pro$_{35S}$:GFP*. Induction of an HR was monitored 48–96 h after transformation.
(b) Leaf disks of tobacco leaves transiently transformed with *Pro$_{35S}$:GFP* (mock) or *Pro$_{35S}$:AtEHD2* were floated on a 250 mM sorbitol solution with or without 2.5 μg ml$^{-1}$ EIX (as indicated) 48 h after transformation. Ethylene biosynthesis was measured after 4 h. Values are the means and SE of four experiments.

were repeated in 20 independent silencing experiments. Thus it would seem that EHD2 has a certain basal level function of endocytosis inhibition in wild-type plants, which can be lost by reducing EHD2 expression.

### AtEHD2 inhibits LeEix signaling but not Pto or FLS2 signaling

We next wished to examine whether AtEHD2 has a similar effect with the ligands of additional pattern recognition receptors (Jones *et al.*, 1994; Takken *et al.*, 1998). FLS2 was chosen as it is the most intensively studied pattern recognition receptor in plants to date, and differs from LeEix2 in that it has an intracellular kinase domain and does not contain a classical endocytosis motif, although it has been shown to undergo endocytosis (Robatzek *et al.*, 2006). Pto was also selected as a control, as it is the product of a cytoplasmic protein kinase *R*-gene (Martin *et al.*, 1993) and its signaling is probably not effected by elicitor internalization. Ethylene biosynthesis induced by EIX is inhibited by AtEHD2 over-expression, while AvrPto- and flg22-induced ethylene biosynthesis is not affected by AtEHD2 over-expression (Figure 7). HR induction by AvrPto was not affected by AtEHD2 over-expression (Figure S4).

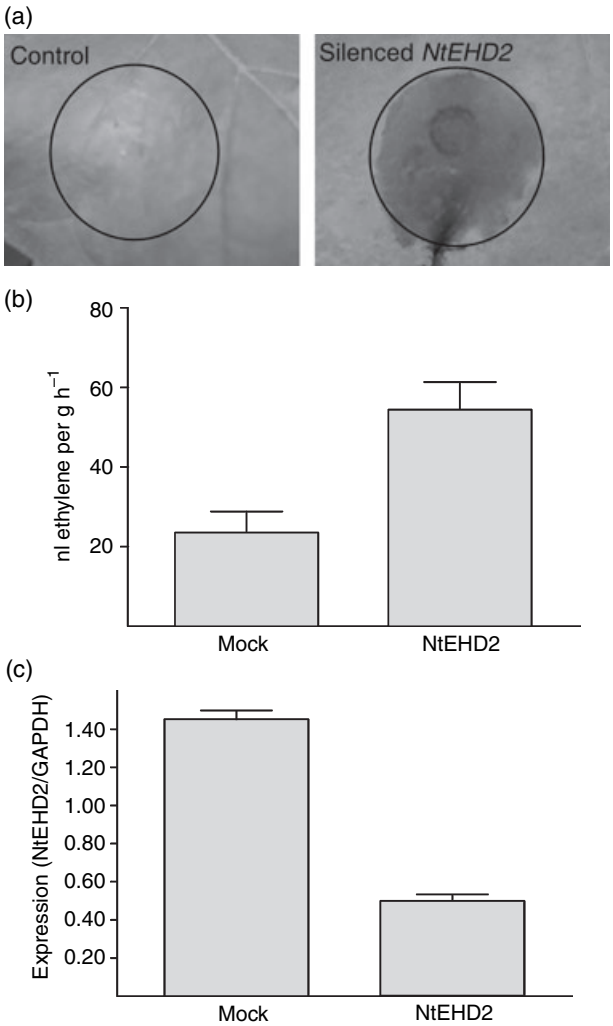


**Figure 6.** Silencing EHD2 enhances induction of an HR and ethylene biosynthesis by EIX.
*Nicotiana benthamiana* plants over-expressing LeEix2 were infected with either TRV (control) or TRV–NtEHD2.
(a) Five weeks post-infection, *Agrobacterium* ($OD_{600}$ = 0.2) harboring *Pro$_{35S}$:tvEix* was injected into the 5th and 6th leaves. HR development was photographed 24 h after injection.
(b) Leaf disks of silenced plants were floated on a 250 mM sorbitol solution with 2.5 μg ml$^{-1}$ EIX. Ethylene biosynthesis was measured after 4 h.
(c) Semi-quantitative RT-PCR was performed on a total cDNA template of silenced plants as indicated. RT-PCR products were separated on an agarose gel stained with ethidium bromide and quantified using IMAGEJ software. The ratio of EHD to GAPDH is presented.
Values are the means and SE of three experiments.

As flg22 does not induce an HR (Hann and Rathjen, 2007), we also examined PR protein synthesis and ROS production, which are additional events 'downstream' of elicitor recognition. PRb-1b was chosen as it has been demonstrated to be induced by EIX treatment (Elbaz *et al.*, 2002). EIX (2.5 μg m$^{-1}$) or flg22 (10 μM) were applied to detached leaves48 h after transient transformation of *N. tabacum* with GFP or AtEHD2. After 4 h, cDNA was prepared from the leaves and *PRb-1b*

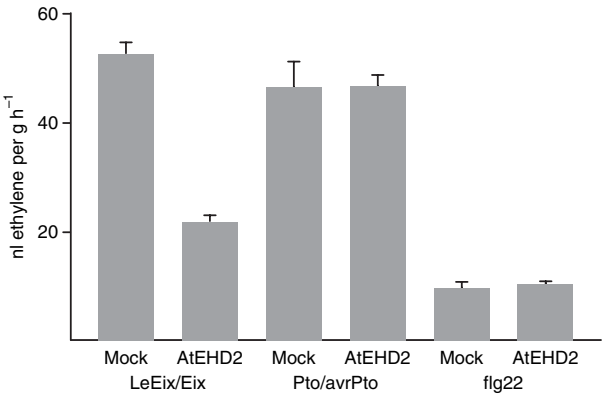


**Figure 7.** EHD2 over-expression selectively inhibits ethylene biosynthesis.
*Nicotiana tabacum* transiently transformed with empty vector (mock) or *Pro$_{35S}$:AtEHD2* and a mixture of *Agrobacterium* containing various combinations of *R* genes and *Avr* genes as indicated. Ethylene production was measured 24 h after transformation with vectors containing LeEix2/EIX or Pto/AvrPto. For FLS2, leaf discs were floated on 10 μM flg22 for 4 h, and ethylene production was monitored. Values are the means and SE of four experiments.

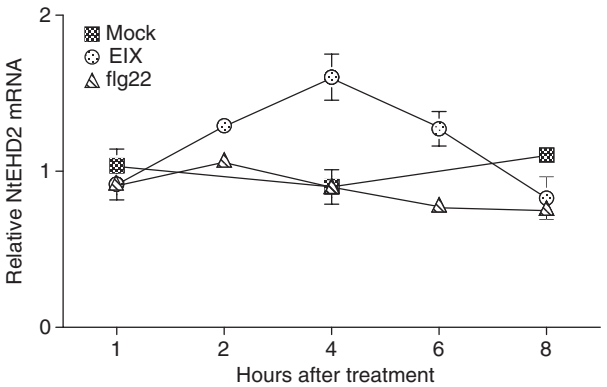


**Figure 9.** Induction of EHD2 gene expression.
Detached *N. tabacum* leaves were incubated for the indicated times in 2.5 μg ml$^{-1}$ EIX or 10 μM flg22 or water. cDNA was prepared, followed by RT-PCR reactions using specific primers to EHD2. RT-PCR products were separated on an agarose gel stained with ethidium bromide and quantified using IMAGEJ software.
Values are the means ± SE of four experiments.

mRNA abundance was examined by semi-quantitative RT-PCR. Figure 8 shows a quantification of four experiments indicating that AtEHD2 inhibits *PRb-1b* mRNA synthesis induced by EIX but does not affect *PRb-1b* mRNA synthesis induced by flg22. Expression of mRNA of the reference gene encoding GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Mayrose *et al.*, 2006) was not affected throughout the experiment (Figure 8). With regard to ROS production, *N. tabacum* over-expressing AtEHD2 or GFP was incubated for 4 h with 2.5 μg ml$^{-1}$ EIX or 10 μM flg22 in the presence of 20 μM 5-amino salicylic acid. Changes in the color of the culture result from a chemical reaction indicating the presence of ROS. AtEHD2 inhibits the formation of ROS as a result of EIX treatment but not as a result of flg22 treatment (Figure S5). The untreated cultures did not change in color throughout the experiment.

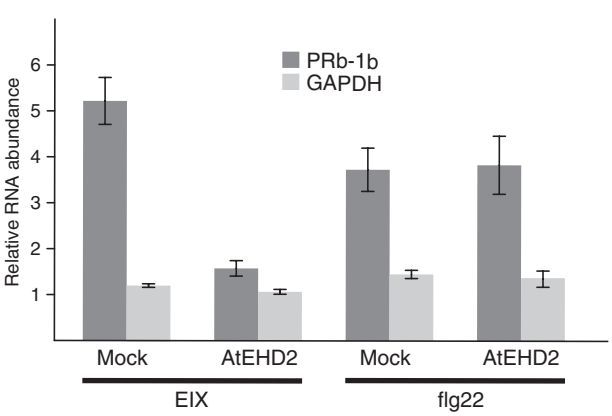


**Figure 8.** EHD2 over-expression selectively inhibits induction of *PRb-1b* mRNA.
*Nicotiana tabacum* leaves were transiently transformed with X (mock) or *Pro$_{35S}$:AtEHD2*. EIX (2.5 μg g$^{-1}$) or flg22 (10 μM) were applied to the petiole of detached leaves 48 h after transformation, and these were then incubated in water for 4 h. cDNA was prepared, followed by RT-PCR reactions using specific primers to PRb-1b or GAPDH. RT-PCR products were separated on an agarose gel, stained with ethiduim bromide, and quantified using IMAGEJ software. Values are the means ± SE of four experiments.

### EIX but not flg22 induces NtEHD2 expression

Microarray data available for AtEHD2 indicate that AtEHD2 expression is induced in response to the *Pseudomonas syringae* elicitor syringolin (Zimmermann *et al.*, 2004; Michel *et al.*, 2006). This raised the interesting possibility that EIX may induce *NtEHD2* expression as part of a feedback/control mechanism. We examined the time course of *NtEHD2* expression for a period of 8 h after EIX and flg22 treatment. Figure 9 shows that *NtEHD2* expression is rapidly induced upon EIX treatment, with a peak of 1.6–1.8 times the basal *NtEHD2* expression at 4 h after EIX application. The induction appears to occur within a narrow time frame, and the level of *NtEHD2* returns to normal 8 h after EIX application. This seems logical, given the detrimental effect that long-term over-expression of EHD2 can have on the plant. By contrast, flg22 does not induce *NtEHD2* expression. Both EIX and flg22 failed to induce expression of the reference gene *GAPDH* (Figure S6).

## DISCUSSION

In this study, we show that endocytosis is a crucial step in the defense response triggered in plants by EIX. Similarly to previous work with FLS2 (Robatzek *et al.*, 2006), we were able to show that signaling by the fungal elicitor EIX is dependent on internalization of its receptor LeEix2 via endocytosis, in a process that requires components of the cytoskeleton. LeEix2 is internalized on highly motile endosomes 15–20 min after EIX application, in a swift

 

endocytic process that follows a similar time course to that described for flg22 (Robatzek *et al.*, 2006), and similar to the time frame of mammalian endocytosis (Gruenberg and Howell, 1987).

According to our results, the endocytic protein EHD2, which we have previously shown to inhibit endocytosis upon over-expression (Bar *et al.*, 2008), appears to be an essential component in endocytosis of the LeEix2 receptor, causing inhibition of the HR and ethylene biosynthesis upon over-expression, but does not seem to be involved in the FLS2 system. EHD2 may affect endocytosis directly, although it is also possible that EHD2 modulates LeEix internalization through an effect on the plasma membrane. LeEix2 is a LRR-RLP and possesses a short cytoplasmic domain containing a Yxx$\phi$ endocytosis motif, while FLS2 is a receptor-like kinase (LRR-RLK) and has an intracellular kinase domain that does not contain a Yxx$\phi$ motif but has instead been reported to contain a non-classical PEST-like endocytosis motif (Gomez-Gomez and Boller, 2000; Robatzek *et al.*, 2006). Another difference between LeEix2 and FLS2, as indicated in this work, is that, while FLS2 appears to be degraded and synthesized *de novo* after flg22-induced internalization, LeEix2 is probably returned (at least in part) to the plasma membrane on recycling vesicles, as indicated by the fact that cycloheximide does not affect its presence in the membrane. The recycling of LeEix2 does not require protein synthesis but may be amplified by synthesis of certain proteins involved.

FLS2 represents the first line of a defense response that recognizes many pathogens (Felix *et al.*, 1999; Gomez-Gomez and Boller, 2000, 2002), and may be regarded as 'broad spectrum', while LeEix2 (Ron and Avni, 2004) represents the second line of defense, which recognizes specific effectors and induces a faster and a stronger response that often culminates in HR induction (Jones and Dangl, 2006). We suggest that the different specificities in signaling may stem from different interacting proteins that mediate the signaling, possibly via different endocytosis pathways, one such endocytic protein being EHD2. It would seem that, while plants have evolved to exhibit similar defense response hallmarks in response to many different MAMPs (Hammond-Kosack and Jones, 1996; Felix *et al.*, 1999; Jones and Dangl, 2006), the signaling pathways that lead to these phenomena may be very different in response to different MAMPs, from the point at which the MAMP is recognized and/or how it enters the plant cell.

Further evidence that EHD2 is specific to the endocytic pathway of LeEix2 but not FLS2 can be gleaned from the fact that EIX but not flg22 induces the expression of NtEHD2. EIX application triggers NtEHD2 expression, upon which NtEHD2 acts to inhibit the defense response in the short term. Longer exposure to the MAMP leads to a 'full-blown' defense response including the HR, free of the inhibitory influence of EHD2, suggesting that a control mechanism based on the interplay of different proteins may be at work. RLKs has specificity to certain signaling pathways in response to certain broad spectrum elicitors via kinase activity (Robatzek *et al.*, 2006). In the case of LeEix2, one could envisage a mechanism in which the MAMP triggers expression of the endocytosis inhibitory protein in order to more tightly control the resultant HR.

The work we present here demonstrates that at least two endocytic mechanisms for pattern recognition receptors exist in plant cells, and that EHD2 is involved in one such mechanism. EHD2 inhibits signaling of LeEix2 (LRR-RLP), probably by inhibiting its endocytosis. The endocytic mechanism of FLS2 appears not to require EHD2 involvement. EHD2 is one molecular component that grants specificity to EIX signaling. Other components will no doubt be identified in the future.

## EXPERIMENTAL PROCEDURES

### Plant and cell culture material and growth conditions

*Nicotiana tabacum* cv. Samsun and *Nicotiana benthamiana* were grown from seeds under greenhouse conditions. *Nicotiana tabacum* cv. Samsun cells were maintained by weekly dilution in fresh MS medium (Murashige and Skoog, 1962) supplemented with 100 $\mu$g $L^{-1}$ 2,4-dichlorophenoxyacetic acid and 30 g $L^{-1}$ sucrose. The cells were maintained with shaking at 110 rpm at 25°C.

### Construction of expression plasmids

*AtEHD1* and *AtEHD2* cDNAs were independently cloned upstream of the *GFP* gene into the *Xba*I site of the binary vector pBINPLUS, generating *Pro$_{35S}$:AtEHD1-GFP* and *Pro$_{35S}$:AtEHD2-GFP*, respectively (Bar *et al.*, 2008). *TvEIX* was cloned into the binary vector pBINPLUS, generating *Pro$_{35S}$:TvEIX* (Ron and Avni, 2004). GFP–LeEix2 was generated using triple template PCR (Tian *et al.*, 2004) and primers P1 (5′-TCGTCGACCCATGGGCAAAAGAACTAATCC-3′), P2 (5′-CCCTGGCTCACCATGGATCCAGCTCCACCTCCACCTCCTCCAAAAGCTGTCTCTAGG-3′), GFP forward (5′-GGAGGTGGAGGTGGAGCTGGATCCATGGTGAGCAAGGGCG-3′), GFP reverse (5′-CGCAGCAGCACCAGCCTTGTACAGCTCGTCCATGCCG-3′), P3 (5′-CGGCATGGACGAGCTGTACAAGGCTGGTGCTGCTGCGTTAACTTCAAGAGAAGTTAACAAGACCC-3′) and P4 (5′-CTGGCGGCCGCTCAGTTCCTTAGCTTTCCC-3′), which positions the GFP between the signal peptide and the rest of the gene, and cloned into the *Sal*I and *Not*I sites of the binary vector pBINPLUS, generating *Pro$_{35S}$:sp-GFP-LeEix2*. The constructs were electroporated into *Agrobacterium tumefaciens* GV3101, and the bacteria were used for transient expression assays. *Agrobacterium tumefaciens* harboring the Pto and AvrPto constructs were obtained from Gregory Martin (The Boyce Thompson Institute for Plant Research, New York).

### Transient transformation

Transient expression was performed as previously described (Ron and Avni, 2004). Briefly, *Agrobacterium* were grown in LB medium overnight, diluted into induction medium (50 mM MES pH 5.6, 0.5% w/v glucose, 1.7 mM $NaH_2PO_4$, 20 mM $NH_4Cl$, 1.2 mM $MgSO_4$, 2 mM KCl, 17 $\mu$M $FeSO_4$, 70 $\mu$M $CaCl_2$ and 200 $\mu$M acetosyringone), and grown for an additional 6 h until the $OD_{600}$ reached 0.4–0.5. The *Agrobacterium* culture was diluted to an $OD_{600}$ of 0.05–0.2, and the suspensions were injected into the leaves of 7–8-week-old tobacco plants. Protein expression in leaves was observed 24–72 h after injection.

### Inhibitor, elicitor and dye applications

Wortmannin and LY294002 (both from Calbiochem, http://www.merckbiosciences.com), latrunculin B (a kind gift from M. Ilan, Zoology Department, Tel Aviv University, Israel) and oryzalin (Sigma, http://www.sigmaaldrich.com/) were applied to detached leaves at final concentrations of 33, 20, 20 and 20 μM, respectively, 20 min prior to EIX application at a final concentration of 2.5 μg $g^{-1}$ tissue (or 2.5 μg $ml^{-1}$ in the case of ethylene assays). EIX (Fluka, http://www.sigmaaldrich.com) was purified as previously described (Dean and Anderson, 1991). Staining of tobacco cells with FM-4-64 was performed as previously described (Bolte *et al.*, 2004; Bloch *et al.*, 2005). *Nicotiana tabacum* cv. Samsun cell cultures were incubated with 5-10 μM FM-4-64 and Hoechst stain (bisbenzimide, Sigma) at a final concentration of 10 μg $ml^{-1}$. flg22 was applied to detached leaves or leaf discs at a final concentration of 10 μM.

### Confocal microscopy

Cells were analyzed using a Zeiss LSM510 Meta confocal laser scanning microscope (Zeiss, http://www.zeiss.com/) with the following configuration: Zeiss LSM510 (30 mW Argon and HeNe lasers, 458, 488, 514, 488 maximum lines HeNe lasers, 541 maximum lines). All images show single sections. The contrast and intensity for each image were manipulated uniformly using Adobe Photoshop (http://www.adobe.com) and/or IMAGEJ software (http://www.rsbweb.nih.gov).

### Bimolecular fluorescence complementation analysis

*LeEix2* cytoplasmic domain (forward primer: 5′-GGGGCCTTTT-AGGCTG-3′; reverse primer P4, as above) and *AtEHD2* cDNA fragments (Bar *et al.*, 2008) were sub-cloned into the *Spe*I site of pSY751, downstream of the N-terminal fragment of YFP (YN), and in pSY752, downstream of the C-terminal fragment of YFP (YC), respectively. The full-length *LeEix2* receptor (both wild-type and Y993A) was sub-cloned into the above vectors in two stages. The signal peptide (forward primer: 5′-TCCCATGGGCAAAAGAACTAATCC-3′; reverse primer: 5′-GGCCATGGTCCAAAAGCTGTCTCTAGG-3′) was cloned into the *Nco*I site upstream of the *YFP* gene fragment, and the gene (forward primer: 5′-GGGTCGACATTAACTTCAAGAGAAG-3′; reverse primer: 5′-CTGGTCGACTCAGTTCCTTAGCTTTCCC-3′) was cloned into the *Sal*I site of both pSY751 and pSY752, downstream of the *YFP* gene fragment (Bracha-Drori *et al.*, 2004). The resulting plasmids, pSY751-LeEix2 (YN–LeEix2), pSY751-LeEix2_CD (YN–LeEix2_CD), pSY751-LeEix2_Y993A (YN–LeEix2_Y993A) and pSY752-AtEHD2 (YC–AtEHD2) were used for transient expression assays in *N. benthamiana*.

### Virus-induced gene silencing assay

Virus-induced gene silencing assays were performed as described previously (Liu *et al.*, 2002b). pTRV1, pTRV2 and pTRV-*tPDS* virus-induced gene silencing vectors (Liu *et al.*, 2002a) were obtained from Dr Dinesh-Kumar (Molecular, Cellular and Developmental Biology, Yale University, CT, USA). A segment of *NtEHD2* (130 bp from bases 494–621) possessing 87% identity to AtEHD2 was amplified using primers NtEHD2 FOR (5′-TGTCACATCAATAGT-TGATGG-3′) and NtEHD2 REV (5′-AGTAAGCAACACTGAAGGTG-3′), and cloned into the pTRV2 vector in the *Xba*I site (Liu *et al.*, 2002a) to generate pTRV2-*NtEHD2*. Five weeks after TRV infection, silenced plants were challenged with $Pro_{35S}$:*tvEIX* (Ron and Avni, 2004).

### Ethylene biosynthesis measurement

Ethylene biosynthesis was assayed as described previously (Avni *et al.*, 1994). Briefly, leaf discs were incubated for 4 h in 250 mM sorbitol and 10 mM MES pH 5.7, supplemented with 2.5 μg $ml^{-1}$ EIX or 10 μM flg22 or not supplemented. The amount of ethylene was measured after 4 h using a gas chromatograph (Varian, http://www.varianinc.com).

### RT-PCR analysis

Total RNA was extracted from leaves of 4–6-week-old *N. benthamiana* silenced plants or detached *N. tabacum* leaves after elicitor treatments as indicated. RNA (0.5–2 μg) was converted to cDNA using M-MLV reverse transcriptase (Promega, http://www.promega.com/). A 2 μl aliquot of each reverse transcriptase reaction was used as a template in a PCR reaction containing the following specific primer pairs: NtEHD2 FOR and NtEHD2 REV (see above), PRb-1b FOR (5′-ATGGGATACTCCACAACATTAGTTGC-3′) and PRb-1b REV (5′-CTAGACATCAGTTGGAAGTTCCAACTTG-3′), and GAPDH FOR (5′-ATGCTCCCATGTTTGTTGTGGGTG-3′) and GAPDH REV (5′-TTAGCCAAAGGTGCAAGGCAGTTC-3′). Quantification of the resultant PCR reactions was performed using IMAGEJ software.

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## SUPPORTING INFORMATION

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

**Figure S1.** *In vivo* functional analysis of GFP–LeEix2 in *N. tabacum* cv. SR1 plants.

**Figure S2.** Time-lapse localization of GFP–LeEix2 with the endosomal marker FYVE–DsRed in leaf tissue.

**Figure S3.** Time lapse co-localization pixel quantification of GFP–LeEix2 with the endosomal marker FYVE–DsRed in leaf tissue.

**Figure S4.** The AvrPto-induced HR is not inhibited by EHD2 over-expression.

**Figure S5.** EHD2 over-expression selectively inhibits induction of ROS.

**Figure S6.** Induction of GAPDH gene expression.

**Movie S1.** Localization of LeEix2 with the endosomal marker FYVE–DsRed in leaf tissue after EIX treatment.

**Movie S2.** Localization of LeEix2 with the endosomal marker FYVE–DsRed in leaf tissue before EIX treatment.

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