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 YxxΦ within the short cytoplasmic domain, FLS2 does not contain a YxxΦ 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 S1). Detached leaves of N. benthamiana transiently transformed with Pro35S:GFP-LeEix2 were treated with EIX (2.5 μg 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 μM) 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
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-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-
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
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. *Pross:tvEix* was transiently expressed with *Pross:AtEHD2*, *Pross:AtEHD2-2* (Bar et al., 2008), *Pross:AtEHD1* (Bar et al., 2008).
is sequentially and structurally similar to AtEHD2 but was found not to inhibit endocytosis upon over-expression [Bar et al., 2008] or Pro35S:GFP (mock treatment). Induction of an HR by EIX was monitored 24–96 h after injection. Leaves transiently expressing a mixture of Pro35S:tvEIX and Pro35S:AtEHD2 or Pro35S:AtEHD2-2 showed no HR (Figure 5a), while leaves transiently expressed a mixture of Pro35S:tvEIX with either Pro35S:AtEHD1 or Pro35S: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 Pro35S:AtEHD2 or Pro35S:GFP. Leaf discs were prepared from the transformed leaves 48 h after transformation and incubated with 2.5 \( \mu \text{g ml}^{-1} \) 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 Pro35S: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 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 \( \mu \text{g g}^{-1} \) 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 \( \mu \text{g ml}^{-1} \)) and the endocytic tracer FM-4-64 (5 \( \mu \text{M} \), 30 min after EIX treatment (2.5 \( \mu \text{g ml}^{-1} \)). 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 \( \mu \text{m} \).
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 affected 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).

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 mL⁻¹) or *flg22* (10 μM) were applied to detached leaves 48 h after transient transformation of *N. tabacum* with GFP or AtEHD2. After 4 h, cDNA was prepared from the leaves and PRb-1b...
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 \( \mu \text{g ml}^{-1} \) EIX or 10 \( \mu \text{M} \) flg22 in the presence of 20 \( \mu \text{M} \) 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.

**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 Yxxφ endocytosis motif, while FLS2 is a receptor-like kinase (RLK-RLK) and has an intracellular kinase domain that does not contain a Yxxφ 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 have 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 μg 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 XbaI site of the binary vector pBINPLUS, generating Pro₃₅S:AtEHD1-GFP and Pro₃₅S:AtEHD2-GFP, respectively (Bar et al., 2008).TvEIX was cloned into the binary vector pBINPLUS, generating Pro₃₅S:TvEIX (Ron and Avni, 2004). GFP-LeEix2 was generated using triple template PCR (Tian et al., 2004) and primers P1 (5¢-TCGTCGACCATGGGCAAAAGAACTAATCC-3¢), P2 (5¢-CCCTTGTGCTCAATGGGAGATTCACCTGC-3¢), P3 (5¢-CAAGCTGCCTCTAGG-3¢), GFP forward (5¢-GGAGGTGGAAGGTG-AGGCTGATCGATCCATGGGAGATTCACCTGC-3¢), P3 (5¢-CGG-GTGAGAGATTCACCTGC-3¢) and primers P4 (5¢-TCGGCCTCTAGG-3¢, which positions the GFP between the signal peptide and the rest of the gene, and cloned into the Sall and Not sites of the binary vector pBINPLUS, generating Pro₃₅S: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 Na₂HPO₄, 20 mM NH₄Cl, 1.2 mM MgSO₄, 2 mM KCl, 17 μM FeSO₄, 70 μM CaCl₂ and 200 μM acetylserine), and grown for an additional 6 h until the OD₆₀₀ reached 0.4–0.5. The Agrobacterium culture was diluted to an OD₆₀₀ 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 (Sigma) at a final concentration of 10 μM 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. fig22 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.rsweb.nih.gov).

Bimolecular fluorescence complementation analysis

LeEix2 cytoplasmic domain (forward primer: 5'-GGGCGCTTTTAGGCTG-3'; reverse primer P4, as above) and AIEHD2 cDNA fragments (Bar et al., 2008) were sub-cloned into the SpeI site of pS7Y51, downstream of the N-terminal fragment of YFP (YN), and in pS7Y52, 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'-TCCCATGGGCAAAGAAGAATAACTAATCC-3'); reverse primer: 5'-GGCATTTGCTCAAAGCTCTGCTTAGG-3') was cloned into the Ncol site upstream of the YFP gene fragment, and the gene (forward primer: 5'-GGTCGACATTAACTTCAAGAGAAG-3'; reverse primer: 5'-CTGGTGACACTCAGTTCTAGTTGTTCGCC-3') was cloned into the SalI site of both pS7Y51 and pS7Y52, downstream of the YFP gene fragment (Bracha-Drori et al., 2004). The resulting plasmids, pS7Y51-LeEix2 (YN-LeEix2), pS7Y51-LeEix2 CD (YN-LeEix2 CD), pS7Y51-LeEix2_Y993A (YN-LeEix2_Y993A) and pS7Y52-AIEHD2 (YC-AIEHD2) 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-IPDS 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 cloned into the pTRV2 vector in the XbaI site (Liu et al., 2002a) to generate pTRV2-NtEHD2. Five weeks after TRV infection, silenced plants were challenged with Pro35S: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 fig22 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: NiEHD2 FOR and NiEHD2 REV (see above), Prb-1b FOR (5'-ATGGGATACTCCACAACTTAGTG-3') and Prb-1b REV (5'-CTAGACATCAGTTGAGAAGTTGACA-3'), and GAPDH FOR (5'-ATGTCCTCATGTGTTGAGGTG-3') and GAPDH REV (5'-TTAGCCAAAGTGCAAGCGGATTTC-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–DesRed in leaf tissue.

Figure S3. Time lapse co-localization pixel quantification of GFP–LeEix2 with the endosomal marker FYVE–DesRed 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.


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