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Endosomal signaling of the tomato leucine-rich repeat receptor-like protein LeEix2

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SUMMARY

Extracellular leucine-rich repeat (LRR) receptor-like proteins (RLPs) represent a unique class of cell-surface receptors, as they lack a functional cytoplasmic domain. Our knowledge of how RLPs that do not contain a kinase or Toll domain function is very limited. The tomato RLP receptor LeEix2 signals to induce defense responses mediated by the fungal protein ethylene-inducing xylanase (EIX). The movement of FYVE-positive endosomes before and after EIX application was examined using spinning disc confocal microscopy. We found that while FYVE-positive endosomes generally observe a random movement pattern, following EIX application a subpopulation of FYVE-positive endosomes follow a directional movement pattern. Further, cellular endosomes travel greater distances at higher speeds following EIX application. Time-course experiments conducted with specific inhibitors demonstrate the involvement of endosomal signaling in EIX-triggered defense responses. Abolishing the existence of endosomes or the endocytic event prevented EIX-induced signaling. Endocytosis/endosome inhibitors, such as Dynasore or 1-butanol, inhibit EIX-induced signaling. Moreover, treatment with Endosidin1, which inhibits an early step in plasma membrane/endosome trafficking, enhances the induction of defense responses by EIX. Our data indicate a distinct endosomal signaling mechanism for induction of defense responses in this RLP system.

Keywords: ethylene-inducing xylanase, endocytosis, signal transduction, endosomal signaling, reactive oxygen species.

INTRODUCTION

Microbial-associated molecular patterns (MAMPs, elicitors) 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). MAMPs have been shown to enter plant cells in several cases, suggesting a potential regulatory role for the endocytic process in their mode of action. In a soybean cell culture, 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). The cryptogein elicitor was shown to induce endocytosis that correlates with its defense response activation (Leborgne-Castel *et al.*, 2008).

The fungal protein ethylene-inducing xylanase (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; Ron *et al.*, 2000). EIX induces ethylene biosynthesis, reactive oxygen species (ROS), medium alkalinization, expression of pathogen related (PR) proteins and hypersensitive response (HR) in specific plant species and/or varieties (Bailey *et al.*, 1990, 1993; Ron *et al.*, 2000; Elbaz *et al.*, 2002; Laxalt *et al.*, 2007). EIX was shown to bind specifically to the plasma membrane of both tomato and tobacco responding cultivars (Hanania and Avni, 1997). The response to EIX in tobacco and tomato cultivars is controlled by a leucine-rich repeat (LRR) receptor-like protein (RLP) encoded by a single dominant locus, termed *LeEix* (Ron and Avni, 2004). The *LeEix* locus contains two functional genes *LeEix1* and *LeEix2. LeEix2* can transmit the signal generated by EIX to induce plant defense responses (Ron and Avni, 2004), while *LeEix1* functions as a decoy receptor (Bar *et al.*, 2010). The amino acid sequence of *LeEix1* and *LeEix2* are 81.4% identical (Ron and Avni, 2004).

Previously we showed that after binding the plant membrane EIX is transported into the cell (Hanania *et al.*, 1999). Mutation in the endocytosis motif of LeEix2 resulted in abolition of induction of HR in response to EIX, which suggested that endocytosis is important in mediating the signal generated by EIX that leads to induction of plant defense responses (Ron and Avni, 2004).

We have also shown that EIX triggers internalization of the LeEix2 receptor and its localization to endosomes (Bar and Avni, 2009). Upon EIX application, EIX binds the LeEix2 receptor on the outside of the plasma membrane (Hanania and Avni, 1997; Ron and Avni, 2004). Ten to 15 min after EIX application the GFP-tagged LeEix2 receptor can be seen throughout the cell on FYVE-positive endosomes (Bar and Avni, 2009). In untreated leaves, GFP-tagged LeEix2 shows much lower co-localization with the FYVE marker. The FYVE domain localizes to endosomes in mammalian cells (Stenmark *et al.*, 1996) as well as in plant cells (Jensen *et al.*, 2001; Heras and Drobak, 2002; Voigt *et al.*, 2005; Vermeer *et al.*, 2006).

Receptor endocytosis has long been recognized as a mechanism to terminate signaling via degradation of activated receptor complexes after their internalization from the cell surface (Chen, 2009). Additionally, in *Drosophila* and mammalian systems it was shown that the endocytic pathway contributes to signaling (Ceresa and Schmid, 2000; Fischer *et al.*, 2006; Murphy *et al.*, 2009; Rajagopalan, 2010). Endocytosis of ligand-activated receptors does not necessarily result in termination of the signaling cascade. In several cases, signals initiated at the plasma membrane continue from endosomes (Rajagopalan, 2010). The knowledge concerning endosomal signaling in plants is very limited (Raikhel and Hicks, 2007; Leborgne-Castel *et al.*, 2010). Some evidence of endosomal signaling in plants was shown in the BRI1 receptor kinase system (Geldner *et al.*, 2007).

In this study the movement of FYVE-positive endosomes before and after EIX application was examined and changes in the speed and displacement parameters monitored. We found that while FYVE-positive endosomes generally observe a random movement pattern, following EIX application a subpopulation of FYVE-positive endosomes follow a directional movement pattern. Time-course experiments conducted with specific inhibitors demonstrate the involvement of endosomal signaling in EIX-triggered defense responses.

RESULTS

Characterization of FYVE-positive endosome distribution and movement in leaf tissue following EIX application

The distribution and movement of FYVE-positive endosomes was examined in the leaf epidermis of *Nicotiana benthamiana* transiently expressing LeEix2 (Ron and Avni, 2004; Bar and Avni, 2009) and FYVE-DsRed (Voigt *et al.*, 2005) following EIX treatment, using a spinning disc confocal microscope or a LSM-510 META confocal microscope and Slidebook™ software (Intelligent Imaging Innovations). To follow endosomes in real time, we acquired time-lapse sequences, comprising 120 frames, typically at 1 frame/826 ms or 2000 ms. Multiple sequences (n = 12) were acquired for each treatment. Over 200 endosomes in total for each treatment were analyzed and scored. Endosomes that appeared in 5–45 frames (23 frames on average) were chosen for analysis. As can be seen in Figure 1, EIX dramatically affects endosomal movement. Treatment with EIX increases endosomal speed (Figure 1a) and displacement (Figure 1b), and shifts the type of movement, in a subpopulation of endosomes, from random movement to directional movement. Thus, while endosomal movement without EIX treatment conforms to a linear equation as a function of the square root of time, an indication of random movement, endosomal movement in a subpopulation of endosomes following EIX treatment conforms to a linear equation that correlates better with time, an indication of directional movement (Figure S1; Ehrlich *et al.*, 2004). EIX increases the average distance from the point of origin, meaning that on average, each endosome will travel greater distances from

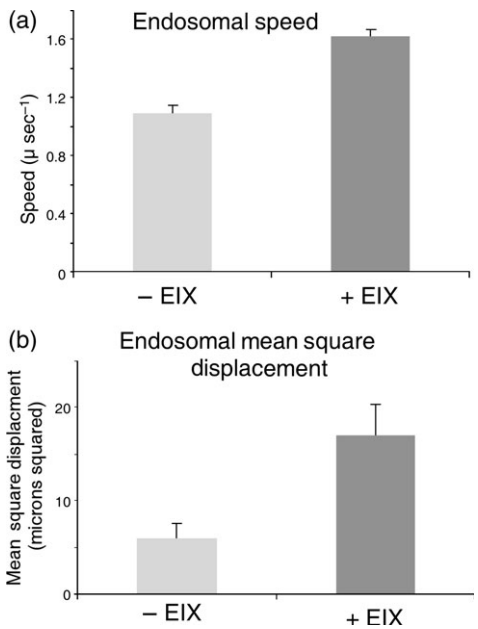

Figure 1. Analysis of FYVE-positive endosome movement parameters in tobacco leaf epidermal cells.
N. benthamiana transiently expressing GFP-LeEix2 and FYVE-DsRed was treated with ethylene-inducing xylanase (EIX) (2.5 μg g^{-1} tissue) and visualized 48 h after transformation. Movies (1 frame per s) were captured using a spinning disc confocal microscope prior to EIX treatment or 10–30 min following EIX treatment.
(a) Endosomal speed.
(b) Endosomal mean square displacement. Over 200 endosomes in more than three movies were analyzed for each sample using Slidebook™ software. Values are the means and standard error (SE) of four experiments.

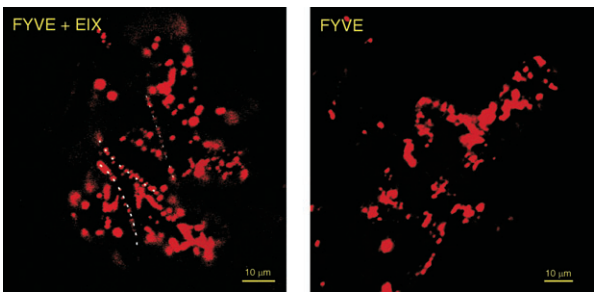

Figure 2. Comparison of collapsed movies prior to and following ethylene-inducing xylanase (EIX) treatment.
N. benthamiana transiently expressing LeEix2 and FYVE-DsRed were treated (left panel) with EIX (2.5 μg g^{-1} tissue) or untreated (right panel) and visualized 48 h after transformation. Movies (1 frame per 2 s) were captured using a Zeiss Laser scanning confocal microscope and collapsed using ImageJ software. Bar = 10 μM. White dots mark directional movement.

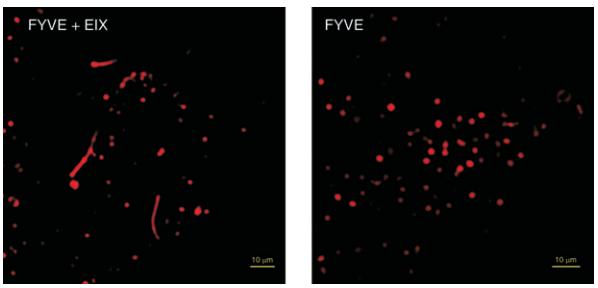

Figure 3. Analysis of FYVE-positive endosome morphology in tobacco leaf epidermal cells.
N. benthamiana transiently expressing LeEix2 and FYVE-DsRed were treated (left panel) with ethylene-inducing xylanase (EIX) (2.5 μg g^{-1} tissue) or untreated (right panel) and visualized 48 h after transformation. Sections were captured using a Zeiss spinning disc confocal microscope and collapsed using Slidebook™ software. Bar = 10 μM.

its point of origin in response to EIX than it will without EIX treatment (Figure 1b).

The directional movement of a subpopulation of FYVE-positive endosomes in response to EIX treatment is also evident in Figure 2, which compares collapsed movies of FYVE-positive endosomes in the absence or presence of EIX, as well as in Movies S1–S3 (Movie S3 shows both LeEix2 and FYVE localization).

Nicotiana benthamiana cells that transiently express tagged FYVE exhibit many endosomes that vary in their size, shape and intensity of localized FYVE. The effect of EIX on endosomal dynamics is perhaps not surprising, as we observed that the percentage of FYVE-positive endosomes that contain transiently expressed LeEix2 increased from an average of 29 ± 2.2% prior to EIX treatment to 91 ± 4% after EIX treatment, demonstrating that the majority of FYVE-positive endosomes internalize LeEix2 in response to EIX treatment. This is also evident in Movie S3. It is however plausible that only part of the FYVE/LeEix2 endosomes observed transmit EIX-induced signaling, as analysis of the movies with and without EIX treatment leads to the observation that only a subpopulation of endosomes are directional in response to EIX, while many endosomes remain random in movement, and yet additional endosomes have very little motility either with or without EIX treatment (Movies S1–S3). The observation that some FYVE-positive endosomes exhibit directional movement following EIX application while others do not, despite the fact that over 90% of FYVE-positive endosomes internalize LeEix2 in response to EIX treatment, seemed to indicate that there are at least two subpopulations of FYVE-positive endosomes that internalize LeEix2. To further assess this we quantified the amount of FYVE (based on pixel intensity) and plotted it against the displacement for each endosome. Figure S2 shows that the displacement (MSD) is inversely related to the FYVE intensity, i.e., the more the endosome moves in relation to its point of origin in response to EIX, the less FYVE it contains. This seems to support the notion that different subpopulations of endosomes internalize LeEix2 in response to EIX. The amount of FYVE localized to different endosomes correlates with specific lipid content (Stenmark *et al.*, 1996; Stenmark and Aasland, 1999).

Following EIX treatment we were able to observe tubular structures stained with FYVE (Figure 3), which were usually absent prior to EIX treatment (Figure 3). Tubular endosomes in mammalian cells are usually characterized as early and/or recycling endosomes (Carlton *et al.*, 2005), and precede pre-vacuolar compartments in the endocytic pathway in plant cells (Lam *et al.*, 2007), likely designating them as early endosomes in plants as well. The increase in abundance of these structures in response to EIX treatment could indicate an increased requirement for early endosomes and/or for recycling. Further experimentation is required to elucidate the function of tubular endosomes in plants.

Inhibitor-based characterization of requirements for EIX-induced signaling

We first examined the effect of various inhibitors on the internalization of LeEix2, as we had done in the past with several additional inhibitors (Bar and Avni, 2009).

1-Butanol, which is widely used as an endocytosis inhibitor (Boucrot *et al.*, 2006), inhibits phospholipase D (PLD) by inhibiting the formation of phosphatidic acid (PA) and forming phosphatidyl-alcohol instead. 1-Butanol prevents the formation of PLD-mediated vesicles and was found to inhibit ligand-induced internalization of epidermal growth factor receptor (EGFR) (Shen *et al.*, 2001). 2-Butanol is used as a control for the effect of 1-Butanol. Dynasore inhibits Dynamin activity, thereby interfering with clathrin-coated vesicle formation (Macia *et al.*, 2006). Brefeldin A (BFA) treatment causes aggregation of endosomal compartments, including the *trans*-Golgi network and early endosomes

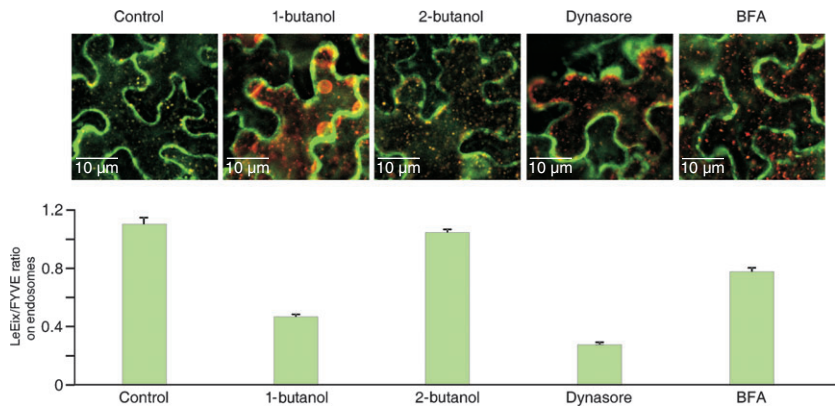

Figure 4. LeEix2 internalization in response to ethylene-inducing xylanase (EIX) treatment in the presence of various inhibitors.
N. benthamiana transiently expressing green fluorescent protein (GFP)-LeEix2 and FYVE-DsRed were treated with inhibitors as indicated and, after 20 min, with EIX (2.5 μg g^{-1} tissue). Sections were captured 10–30 min following EIX treatment using a Zeiss Laser scanning confocal microscope. Bar = 10 μM. For LeEix2/FYVE ratio, the content of 60–120 cellular endosomes in each of three to five separate cells (in different sections) was quantified and analyzed using Slidebook™ software. Values are the means and standard error (SE) of three experiments. BFA, brefeldin A.

(Grebe *et al.*, 2003; Dettmer *et al.*, 2006; Robinson *et al.*, 2008a). It is commonly considered a recycling inhibitor (Amenos *et al.*, 2009), as it usually prevents recycling vesicle trafficking due to fragmentation of the Golgi.

As shown in Figure 4, Dynasore and 1-butanol inhibit LeEix2 internalization in the presence of EIX. BFA also attenuates the internalization but to a lesser degree and 2-butanol does not affect it. Figure 4 (bottom panel) presents the ratio of a quantification of the endosomal content of LeEix2 and FYVE in the various treatments as indicated (LeEix2 pixels/FYVE pixels).

Binding experiments were performed to examine the effect of these inhibitors on EIX binding and rule out the possibility that endocytosis inhibitors affect the binding of EIX to LeEix2 on the cell membrane. Fluorescein isothiocyanate (FITC)-EIX was previously shown to bind specifically to *N. tabacum* cv. Xanthi cells in suspension, requiring only the presence of LeEix2 to do so (Hanania and Avni, 1997; Rotblat *et al.*, 2002; Ron and Avni, 2004). *N. tabacum* cv. Xanthi cells were treated with the above inhibitors for 20 min followed by incubation with FITC-labeled EIX (0.5 μg ml^{-1}). Cells were examined by fluorescence microscopy 15 min after FITC-EIX application (Figure 5). As shown in Figure 5, treatment with the endocytosis inhibitors did not affect the ability of EIX to bind tobacco cells, but destroyed the ability of the cell to internalize LeEix2 in the case of Dynasore and 1-butanol (Figure 4).

We examined the induction of cell death in response to EIX in *N. tabacum* cell cultures in the presence of various inhibitors. Cell death in *N. tabacum* cv. Xanthi cultures has been characterized by us in the past (Elbaz *et al.*, 2002) and is a reliable indicator of the amplitude of the cellular response to EIX.

Wortmannin and LY294002 inhibit internalization of LeEix2 on FYVE-positive endosomes in response to EIX treatment (Bar and Avni, 2009). Wortmannin is considered to be a specific inhibitor of phosphoinositide-3-kinases (PI3Ks), but it has been shown to inhibit additional associated proteins

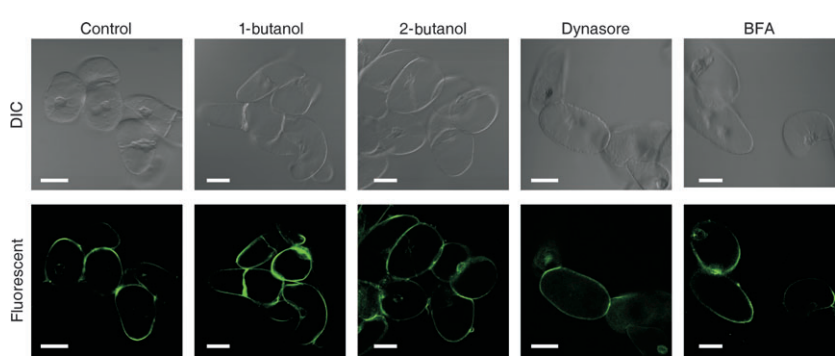

Figure 5. Binding of ethylene-inducing xylanase (EIX)–fluorescein isothiocyanate (FITC) to cultured tobacco cells in the presence of various inhibitors.
N. tabacum cv. Xanthi cell culture was treated with EIX-FITC (0.5 μg ml^{-1}). Cells were treated with inhibitors as indicated 20 min prior to EIX-FITC application. Cells were visualized using a Zeiss LSM-510 confocal laser scanning microscope (Zeiss) (488 nm excitation) 15 min after FITC-EIX application. BFA, brefeldin A. Bar = 20 μM.

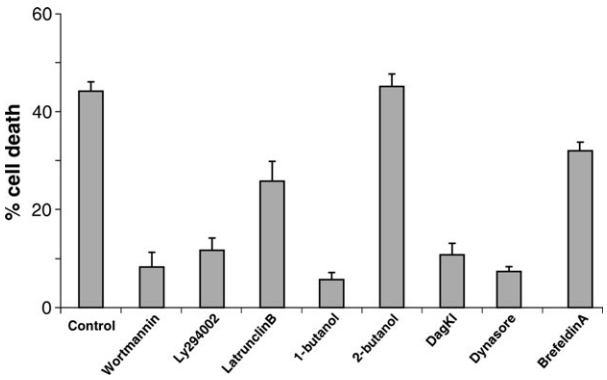

Figure 6. Cell death in response to ethylene-inducing xylanase (EIX) treatment in the presence of various inhibitors.
N. tabacum cv. Xanthi cell culture was treated with EIX (2.5 μg ml^{-1}). Cells were treated with inhibitors as indicated 20 min prior to EIX application. Over 200 cells in four different experiments were examined 4 h after EIX application. Values are the means and standard error (SE) of four experiments.

(Emans *et al.*, 2002) such as phosphoinositide-4-kinase (PI4K). LY294002 was reported to be a selective PI3K inhibitor (Vlahos *et al.*, 1994), and is considered a more 'narrow' endocytosis inhibitor than Wortmannin. As was demonstrated for internalization, we found that Wortmannin and LY294002 also inhibit cell death in response to EIX (Figure 6). The same was found for the actin inhibitor Latrunculin B (Figure 6). As shown in Figure 6, 1-butanol inhibits cell death in response to EIX, while the secondary alcohol 2-butanol, used as a control, does not.

Diacyl-glycerol kinase inhibitor (DAGKI) shifts the balance in the PLC/PLD pathway and affects endocytosis and vesicle trafficking by influencing the lipid/phosphatidyl synthesis pathway (Chen *et al.*, 2007). We found that DAGKI suppresses cell death in response to EIX treatment, indicating that PLC activity is also crucial for EIX-induced signaling (Figure 6).

We examined the importance of Dynamin activity in EIX internalization/signaling by examining cell death in response to EIX in the presence of Dynasore, and found cell death to be greatly inhibited (Figure 6), indicating Dynamin activity to be crucial for the internalization and/or signaling of EIX.

We examined the effect of BFA on cell death in response to EIX and found that BFA treatment inhibits cell death caused by EIX to a certain extent. This could indicate that BFA has a broader effect than as a mere recycling inhibitor. The primary target of BFA is most likely a Golgi ARF-GEF. However, the stochastic distribution of ARF-GEF with differing sensitivity to BFA could indicate a non-uniform response to BFA treatment in different plants (Ritzenthaler *et al.*, 2002; Robinson *et al.*, 2008b; Lam *et al.*, 2009). BFA was shown to affect compartments that mediate trafficking of other plasma membrane proteins and thus has a pleotropic effect in plant cells (Geldner *et al.*, 2001).

The involvement of signaling endosomes in the EIX/LeEix system was also examined by looking at gene transcription and ROS production in a short, internalization-relevant time frame. EIX was shown to induce ethylene biosynthesis (Avni *et al.*, 1994) and ROS production (Laxalt *et al.*, 2007; Bar and Avni, 2009; Yordanova *et al.*, 2009). One of the first steps in ethylene biosynthesis is an increase in the mRNA of the 1-aminocyclopropane-1-carboxylate (ACC) synthase gene, an ethylene biosynthesis enzyme, which occurs as early as 30 min after EIX treatment (Avni *et al.*, 1994). As the peak of LeEix2 endocytosis occurs 20–30 min after EIX application (Bar and Avni, 2009), inhibition of the increase in ACC synthase mRNA by endocytosis inhibitors can provide additional supporting evidence for the existence of signaling endosomes in the EIX-induced signaling pathway. Quantitative polymerase chain reaction (qPCR) on cDNA prepared from mRNA of cell cultures 30 min after treatment with EIX in the presence of various inhibitors (Figure 7a)

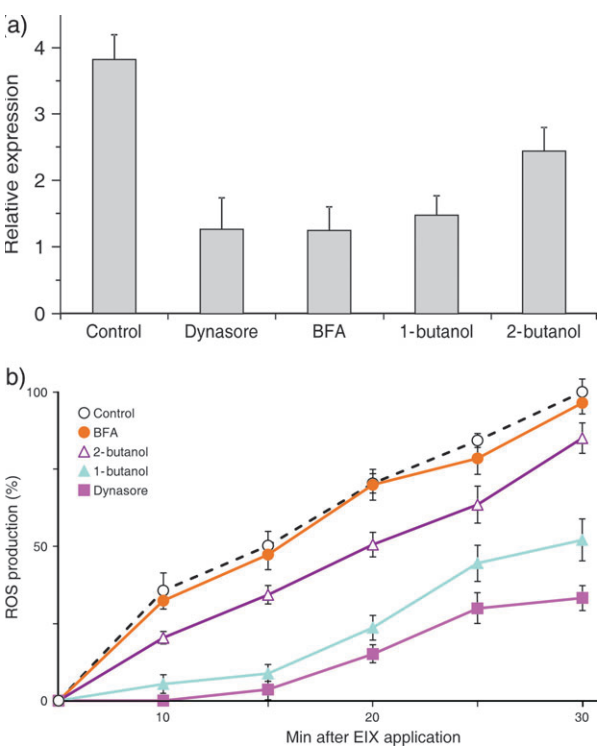

Figure 7. Induction of ACC synthase gene expression and ROS production.
N. tabacum cv. Xanthi cells were incubated for 20 min with various inhibitors (as indicated); 2.5 μg ml^{-1} ethylene-inducing xylanase (EIX) was then added.
(a) cDNA was prepared followed by quantitative reverse transcription polymerase chain reactions (qPCR) using ACC synthase specific primers. Relative expression of ACC synthase compared with untreated cells is presented. Each point represents the average ± standard error (SE) of three replicates.
(b) Cells were incubated in the presence of 5-amino salicylic acid (100 μM) for different time points as indicated. Changes in the color of the culture result from a chemical reaction indicating the presence of reactive oxygen species (ROS). Images were quantified using ImageJ software. Each point represents the average ± SE of four different experiments. BFA, brefeldin A.

demonstrates that the endocytosis inhibitors Dynasore and 1-butanol inhibit the increase in ACC synthase mRNA abundance in response to EIX. ROS production was monitored between 10 to 30 min after application of EIX in the presence of various inhibitors in *N. tabacum* cv. Xanthi cells. Dynasore and 1-butanol treatment significantly inhibited ROS production (Figure 7b). Pre-treatment with BFA inhibited the induction of ACC synthase mRNA (Figure 7a), while it did not affect ROS production (Figure 7b).

Genetic interference with the clathrin machinery impairs LeEix signaling

Previously we showed that mutagenizing the endocytosis signal in LeEix2 inhibits EIX-induced endocytosis and signaling (Ron and Avni, 2004; Bar and Avni, 2009). We also showed that overexpression of EHD2 inhibits LeEix2 signaling (Bar and Avni, 2009). Here we show that overexpression of the clathrin heavy chain Hub1 domain, which inhibits endocytosis of FM 4-64 and PIN1 (Dhonukshe *et al.*, 2007) also inhibits LeEix2 signaling (Figure 8). The Hub1 domain was over-expressed in *N. tabacum* cv. Xanthi cells (EIX responsive cells) under the control of the 35S promoter. Overexpression of Hub1 inhibits cell death in response to EIX treatment (Figure 8a). Overexpression of Hub1 in *N. tabacum* cv. Xanthi leaves also inhibits the induction of electrolyte leakage by EIX as compared control leaves treated with EIX (Figure 8b). Above we present results demonstrating pharmacological inhibition of endocytosis. Use of two different genetic systems (overexpression of EHD2 and the clathrin heavy chain Hub1 domain) which confer a dominant negative effect on endocytosis (Dhonukshe *et al.*, 2007; Bar and Avni, 2009) further demonstrates that endocytosis is required for EIX-induced signaling.

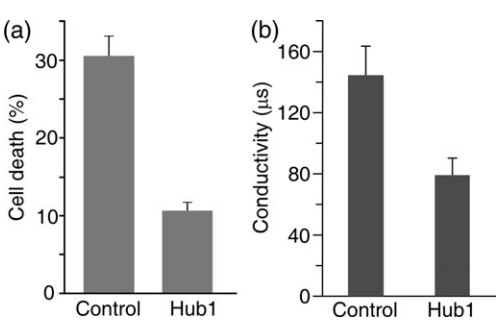

Figure 8. Genetic interference with the clathrin machinery impairs LeEix signaling

(a) *N. tabacum* cv. Xanthi cultured cells were transiently transformed with the dominant negative clathrin heavy chain Hub1 domain. Cells transiently transformed with GFP served as a control. Cells were treated with ethylene-inducing xylanase (EIX) (2.5 μg ml^{-1}). Over 300 cells in three different experiments were examined 2 h after EIX application. Values are the means and standard error (SE) of three experiments.

(b) *N. tabacum* cv. Xanthi leaf epidermal cells were transiently transformed with clathrin heavy chain Hub1 domain. Cells transiently transformed with GFP served as a control. Leaves were treated with EIX (2.5 μg g^{-1} tissue). Ion leakage was measured 24 h post-EIX application. Values are the means and SE of three experiments.

Examination of endosomal participation in EIX-induced signaling

The LeEix2 receptor must be internalized for EIX-induced signaling to occur, suggesting that at least part of the signaling pathway may originate in endosomes. Signaling endosomes have been suggested to exist in plant cells (Geldner *et al.*, 2007). To further characterize EIX-induced signaling we chose to continue examining the effect of the PLD and Dynamin endocytosis inhibitors 1-butanol and Dynasore and the recycling inhibitor BFA. In order to examine whether EIX-induced signaling can occur from endosomal structures in plants, we tested these inhibitors in two time frames. The inhibitors were either applied 20 min prior to EIX treatment, as above, referred to herein as 'time zero' or '−20 min' treatment, or 45 min after EIX application, whereupon internalization of LeEix2 has already occurred. Endocytic markers were shown to enter suspended plant cells within 10–30 min (Bolte *et al.*, 2004; Jelinkova *et al.*, 2010). If the endosomal structures are only needed for internalization, treatment with these inhibitors in an internalization-irrelevant time frame should not affect EIX-induced signaling. As shown in Figure 9, treatment of cell cultures with 1-butanol or Dynasore 20 min prior to EIX treatment inhibits EIX-induced cell death, as well as EIX-induced medium alkalinization and ethylene production. The effect of BFA on EIX-induced signaling remained mostly unchanged in the time frame of the experiment. Dynasore treatment 45 min following EIX application failed to inhibit EIX-induced signaling (Figure 9), as expected, as Dynamin is required for internalization of the endocytic vesicle. 1-Butanol continued to inhibit EIX-induced signaling even when applied 45 min after EIX application, though to a significantly lesser degree than if given 20 min prior to EIX (Figure 9). This may indicate that some of the EIX-derived signaling originates from endosomal compartments which are sensitive to PLD inhibition. 2-Butanol did not influence EIX-induced signaling, irrespective of the time of application.

Endosidin1 vesicle movement inhibitor enhances EIX-induced signaling

Endocytosis of several plant receptor-like kinases (RLK) was shown to be BFA sensitive (Russinova *et al.*, 2004; Gifford *et al.*, 2005). BFA was able to enhance BRI1-dependent signaling (Geldner *et al.*, 2007). However, BFA did not enhance the signal for induction of ethylene biosynthesis or ROS production mediated by EIX. The bioactive limonoid endosidin1 (ES1) inhibits trafficking at the early endosome (Robert *et al.*, 2008). ES1 interferes selectively with endocytosis in seedlings, providing a unique tool to dissect recycling pathways (Robert *et al.*, 2008). ES1 selectively disrupted the trafficking of PIN2, AUX1, and BRI1 but not PIN1 and PIN7, suggesting at least two pathways for endo-

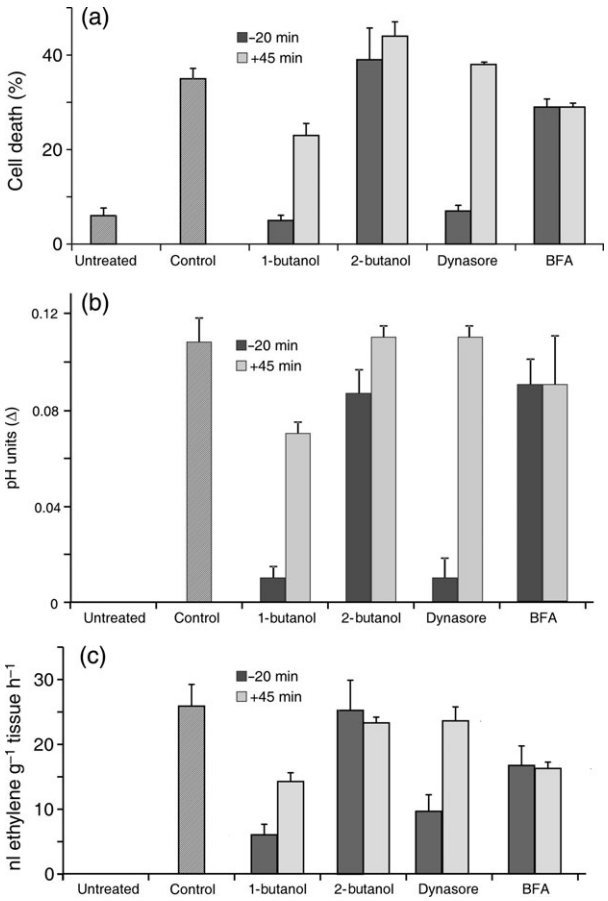

Figure 9. Induction of defense responses mediated by ethylene-inducing xylanase (EIX) in the presence of various inhibitors applied at different time points.
(a) *N. tabacum* cv. Xanthi cell culture was treated with EIX (2.5 μg ml^{-1}). Cells were treated with inhibitors as indicated 20 min prior or 45 min after EIX application. Over 200 cells in four different experiments were examined 4 h after EIX application. Values are the means and standard error (SE) of four experiments.
(b) *N. tabacum* cv. Samsun leaf discs were treated with EIX (2.5 μg ml^{-1}) and inhibitors as indicated 20 min prior to or 45 min after addition of EIX. Changes in medium pH were monitored 4 h after treatment. Changes in pH as compared with untreated leaf discs are presented. Each point represents the average ± SE of three different experiments.
(c) *N. tabacum* cv. Samsun leaf discs were treated with EIX (2.5 μg ml^{-1}) and inhibitors as indicated 20 min prior to or 45 min after addition of EIX. Ethylene production in response to EIX treatment was monitored 4 h after treatment. Each point represents the average ± SE of three different experiments. BFA, brefeldin A.

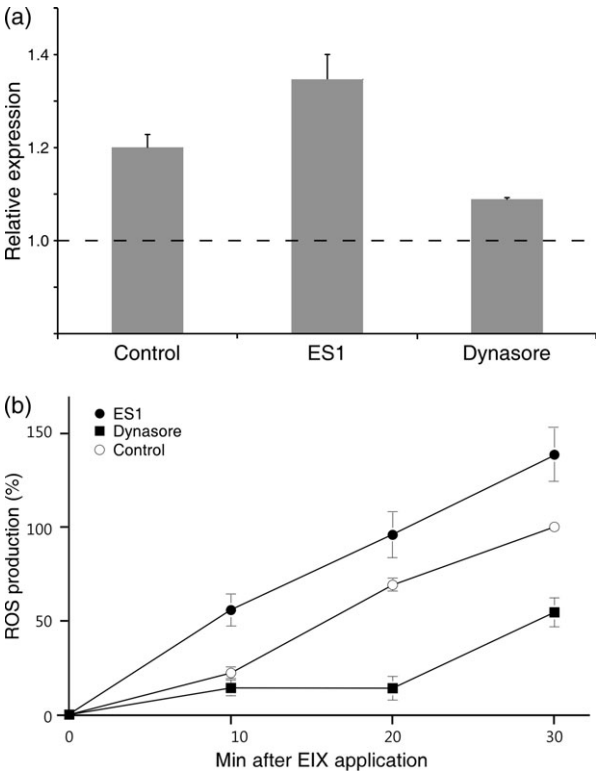

Figure 10. The effect of endosidin1 (ES1) treatment on the induction defense responses mediated by ethylene-inducing xylanase (EIX).
N. tabacum cv. Xanthi cells were incubated for 2 h with ES1 or, 20 min with Dynasore followed by the addition of 2.5 μg ml^{-1} EIX.
(a) cDNA was prepared 30 min after EIX application followed by semi-quantitative reverse transcription polymerase chain reactions (RT-PCR) using specific primers to 1-aminocyclopropane-1-carboxylate (ACC) synthase. RT-PCR products were separated on an agarose gel, stained with ethidium bromide and quantified using ImageJ software. The amount of ACC synthase transcript in untreated cells was set to 1.0. Relative expression of ACC synthase compared to untreated cells is presented. Each point represents the average ± standard error (SE) of three different experiments.
(b) Cells were incubated in the presence of 5-amino salicylic acid (100 μM) for different time points as indicated. Changes in the color of the culture resulted from a chemical reaction that indicated the presence of reactive oxygen species (ROS). Images were quantified using ImageJ software. Each point represents the average ± SE of four different experiments.

cytosis or recycling (Robert *et al.*, 2008). In order to further examine EIX-induced signaling upon an arrest of endosomal traffic, we tested the effect of ES1 treatment on the induction of ACC synthase transcription and ROS production after EIX treatment. *N. tabacum* cells were incubated for 2 h with ES1 or Dynasore as described above followed by treatment with EIX (2.5 μg ml^{-1}). Semi-quantitative PCR shows that Dynasore inhibits ACC synthase expression, while ES1 treatment enhances the induction of ACC synthase compared to the control (Figure 10a). The formation of ROS was monitored after EIX treatment in the presence of Dynasore or ES1 (Figure 10b). ES1-treated cells induced higher level of ROS as compared with the control (Figure 10b). The untreated cultures did not produce ROS throughout the experiment. In summary, ES1 treatment enhances EIX-dependent signaling. Our data indicate that LeEix2 signals from endosomes.

DISCUSSION

Endocytosis has emerged in recent years as a requirement for defense signaling in plants (Leborgne-Castel *et al.*, 2010). In particular, it has been reported that defense receptor internalization is required for proper defense signaling (Ron and Avni, 2004; Robatzek *et al.*, 2006; Bar and Avni, 2009). As we have previously reported, internalization of the LeEix2

receptor is required for the plant to mount a proper response to EIX (Ron and Avni, 2004; Bar and Avni, 2009). We have previously demonstrated that inhibition of PI3-kinase using Wortmannin or LY294002 prevents internalization of the LeEix2 receptor (Bar and Avni, 2009), and we show here that this inhibition also prevents proper EIX-induced signaling. Prevention of internalization of endocytic vesicles by inhibition of Dynamin also impaired EIX-induced signaling, further strengthening the evidence of internalization being an obligatory requirement for EIX/LeEix2 signaling and defense responses.

Characterization of endosomal movement following EIX treatment allowed us to demonstrate that EIX causes endosomes to move faster and to greater distances. A subpopulation of endosomes also exhibit directional movement following EIX treatment. Analysis of endosomal content demonstrated that there is roughly a 1:1 FYVE:LeEix2 ratio on an average of 91% of cellular endosomes following EIX application. As not all endosomes possess directional movement following EIX application, it would seem that while almost all FYVE-positive endosomes contain LeEix2, only a subpopulation of these endosomes respond to EIX application in this manner. Further, endosomes which contain a smaller amount of FYVE exhibit greater displacement in response to EIX than endosomes which contain higher amounts of FYVE, seeming to indicate that there are different endosomal classes (which contain LeEix2 in response to EIX), and, as discussed above, not all classes possess similar movement. Directional movement in response to a MAMP/elicitor could stem from targeting to particular cellular organelles, said targeting being a component of the plant defense response or a mechanism originating from the pathogen or organism from which the elicitor is derived. Interestingly, reorganization of cytoskeleton and vesicle trafficking was demonstrated in the interaction between plant cells and microorganisms (Takemoto *et al.*, 2003; Takemoto and Hardham, 2004; Lipka and Panstruga, 2005). Given the requirement of an intact cytoskeleton for EIX-induced signaling, it seems probable that directional movement following EIX treatment occurs on actin fibers. The changes in endosomal content following EIX treatment may also indicate that a particular subpopulation of cellular endosomes is involved in EIX/LeEix2 transport and signaling. Though endosomal compartments are not well characterized in plants and the term 'endosome' is often employed when relating to any intracellular vesicle containing internalized material, it is clear that EIX treatment leads to enrichment in endosomes which are directional as well as in tubular endosomes.

Given the requirement of endocytosis of the receptor for proper EIX-induced defense responses to occur, it is perhaps not surprising that components of membrane lipid metabolism and signaling are involved in the plant's response to EIX. Impairing PLD or PLC activity impairs membranal integrity and ultimately leads to inability of the cell to form endosomes, which in turn prevents endocytosis. Additionally, certain products of membrane lipid metabolism such as PA may themselves serve as second messengers (Laxalt *et al.*, 2007; Vossen *et al.*, 2010) and be involved in EIX/LeEix2 signaling.

Cell-surface signaling is transient in nature (seconds to minutes), while endosomal signaling can sustain for a longer-term (minutes to hours) by the increased residence of the activated receptor in endosomes (Rajagopalan, 2010). The prevailing view that signaling occurs on the plasma membrane only was challenged in the early 1990s by Bergeron, Posner and colleagues, who observed that shortly after ligand addition the majority of activated EGFRs and their downstream signaling factors such as Shc, Grb2 and mSOS were found not on the plasma membrane but on early endosomes (Di Guglielmo *et al.*, 1994), suggesting that EGFR signaling continues from this compartment (Baass *et al.*, 1995). Evidence of signaling endosomes has previously been reported in plants (Geldner *et al.*, 2007). In this example treatment with BFA was able to enhance BRI-dependent signaling. However, BFA could not enhance the signal for induction of ethylene biosynthesis or ROS production mediated by EIX. The decrease in signaling upon BFA treatment irrespective of the time frame seems to suggest that BFA may cause abrogation of one of the required downstream endosomal compartments.

In cultured cells of tobacco, BFA interferes with ARF-GEF-dependent endoplasmic reticulum (ER)-to-Golgi trafficking, leading to ER–Golgi hybrids (Ritzenthaler *et al.*, 2002). In contrast, in Arabidopsis, this process is catalyzed by the BFA-resistant ARF-GEF GNOM-LIKE1 (GNL1) (Richter *et al.*, 2007). The prominent BFA target in Arabidopsis is the endosomal ARF-GEF GNOM, which mediates mainly endosomal recycling to the plasma membrane, whereas endocytosis from the plasma membrane seems to remain operational (Geldner *et al.*, 2003). This difference in mode of action of BFA between Arabidopsis and tobacco may explain the inability of BFA to enhance the EIX signal.

Abolishing endocytosis caused a complete arrest in EIX-induced signaling, indicating that LeEix/EIX cannot transmit a signal from the cell surface and must be internalized in order to do so. ES1 was shown to block the endocytosis of several PM auxin transporters and appears to display more compartmental specificity in its action than BFA (Robert *et al.*, 2008). We used ES1 as an alternative vesicle transport inhibitor to BFA. We found that treatment with ES1 enhances EIX/LeEix2-dependent signaling, again suggesting that LeEix2 signals from endosomes.

As EIX-derived signaling occurs as early as 5–10 min after the addition of EIX, it is highly likely that at least some of the signal is transmitted directly from endosomes. Preventing the formation of endosomes after internalization occurred caused a decrease in EIX-induced signaling, while interfer-

ing with vesicle trafficking by ES1 enhanced EIX-induced signaling, further supporting the notion of endosomal signaling. Additional exploration of endosomal signaling in the EIX/LeEix2 system and in plants in general is required in order to further characterize plant signaling endosomes.

EXPERIMENTAL PROCEDURES

Plant and cell culture material and growth conditions

N. tabacum cv. Xanthi and *N. benthamiana* were grown from seeds under greenhouse conditions. *N. tabacum* cv. Xanthi cells (Hanania and Avni, 1997) were maintained by weekly dilution in fresh MS medium (Murashige and Skoog, 1962) supplemented with 0.2 mg L^{-1} 2-4-D. The cells were maintained in the dark with shaking at 110 rpm at 25°C.

Transient transformation

Transient expression was performed as previously described (Ron and Avni, 2004). Briefly, *Agrobacterium* of strain GV3101 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 μM $FeSO_4$, 70 μM $CaCl_2$ and 200 μM acetosyringone), and grown for an additional 3–6 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.

Construction of expression plasmids

FYVE-DsRed (Voigt *et al.*, 2005) was obtained from Dr Jozef Samaj. GFP-LeEix2 was cloned as described in (Bar and Avni, 2009). The clathrin heavy chain Hub1 was obtained from Drs Friml and Dhonukshe (Dhonukshe *et al.*, 2007). The Hub1 fragment was cloned into the binary vector Bin19 and *Agrobacterium* GV3101 was used for transient overexpression in *N. tabacum* cv. Xanthi cells and leaves.

Inhibitor and elicitor application

Wortmannin (33 μM; Calbiochem, http://www.calbiochem.com), LY294002 (20 μm; Calbiochem), Latrunculin B (20 μM; a gift from Prof. M. Ilan, Zoology Department, Tel Aviv University, Israel), Brefeldin A (50 μM; Sigma, http://www.sigmaaldrich.com), Dynasore (100 μM; Sigma), 1-butanol (0.8% v/v; Sigma), 2-butanol (0.8% v/v; Sigma), diacyl-glycerol kinase inhibitor (30 μM; Sigma), 5-ASA (100 μM; Sigma) and endosidin1 (16 μM; a gift from Prof. N. Raikhel, UC Riverside, USA) were added to the *N. tabacum* cv. Xanthi cell cultures as indicated, 20 min prior to EIX application or 45 min after EIX application. EIX was purified from Sigma crude xylanase extract as previously reported (Dean and Anderson, 1991) and applied to cell cultures or leaf discs at a final concentration of 2.5 μg ml^{-1} or applied to the petiole of detached leaves at a final concentration of 2.5 μg g^{-1} tissue. EIX–FITC was prepared using FITC purchased from Molecular Probes. EIX purified as described above was labeled with FITC essentially according to the manufacturer's instructions; EIX was incubated with FITC for 7 days with gentle shaking and dialysis purified.

Microscopy

Data on endosomal movement obtained from movies taken with a Zeiss spinning disc confocal microscope or a Zeiss LSM META confocal microscope were analyzed using Slidebook™ software. Spinning disc images were captured with a microscope setup consisting of a 200M microscope (Zeiss, http://www.zeiss.com/), a CSU-22 spinning disk (Yokogawa, http://www.yokogawa.com/), solid-state lasers (473 and 561 nm, cobalt), a 1.4 NA × 63 lens (Zeiss), and an HQ2 CCD (Photometrics, http://www.photometrics.com). Typical exposure times were 500 ms. Additional images were captured with a Zeiss LSM-510-Meta confocal laser scanning microscope (Zeiss) with the following configuration: 30 mW Argon and HeNe lasers, 458, 477, 488, 514 and 568 maximum lines respectively. Typical exposure times were of 826 or 2000 ms. All images depict single sections, except when indicated otherwise. Contrast and intensity for each image were manipulated uniformly using Adobe Photoshop and/or ImageJ software (http://rsbweb.nih.gov).

Cell death, ethylene ROS and ion leakage measurements

Cell death was determined morphologically by counting at least 200 cells from each sample, as previously described (Elbaz *et al.*, 2002). Ethylene biosynthesis was measured with a gas chromatograph 4 h post-EIX application as previously described (Bar and Avni, 2009). ROS were quantified using 100 μM of 5-amino salicylic acid 5–30 min following EIX application to 1 ml of *N. tabacum* cv. Xanthi cell culture. Changes in the color of the culture result from a chemical reaction indicating the presence of ROS. Pixels were quantified with ImageJ software.

Ion leakage was measured 24 h post-EIX application. Six leaf discs (1 cm diameter) of *N. tabacum* cv. Xanthi were sampled from treated areas for each plant and floated in 3 ml double-distilled water (DDW) with gentle shaking. Conductivity was measured with a Eutech Con510 instrument (Eutech Instrument, http://www.eutechinst.com).

RNA isolation, quantitative and semi-quantitative RT-PCR analysis

Total RNA was extracted from 2 ml of 3–5-day-old *Nicotiana tabacum* cv. Xanthi cell cultures (25–35% packed volume) treated as indicated using the SV Total RNA Isolation System (Promega, http://www.promega.com) according to manufacturer's instructions.

RNA samples (2 μg) were reverse-transcribed for 50 min at 42°C in a 30-μl reaction volume containing 100 units of SuperScript II $RNaseH^-$ reverse transcriptase (Invitrogen, http://www.invitrogen.com/), 10 mM dithiothreitol, 1 × RT buffer (Invitrogen), 40 U of RNase inhibitor (Invitrogen), 500 μM of each dNTP and 500 ng oligo$(dT)_{18-24}$ primer. The reaction was stopped by incubation at 70°C for 15 min. Gene-specific primers for the analyzed genes were designed to have a melting temperature of 60 ± 3°C, by means of the Primer 3 program of the Biology WorkBench software package (http://workbench.sdsc.edu/). The PCR efficiency was determined for each set of primers by using standard curves with six cDNA dilutions (1:5, 1:10, 1:25, 1:50, 1:125, 1:500). PCR reactions were performed in triplicates and contained template cDNA, 200 nM gene-specific primers, and Power SYBR® Green PCR Master Mix (Applied Biosystems, https://products.appliedbiosystems.com) in a volume of 15 μl. Reactions were carried out with an Mx3000P QPCR system (Stratagene, http://www.genomics.agilent.com) with the following cycling program: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Fluorescence was measured at the end of each cycle. The absence of non-specific products and primer dimers was confirmed by analysis of melting curves. For data analysis, average threshold cycle (C_T) values were calculated for each gene of interest, on the basis of two independent biological samples, normalized and used to calculate relative transcript levels as described (Pfaffl, 2001). The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene from *N. benthamiana* (SGN accession no. SGN-E1199677) was used as internal standard

for normalization. Primer pairs were as follows: GAPDH FOR: 5′-CAGTGCTGCTAGGAATGATGTTG-3′; GAPDH REV: 5′-TCATGACTACTGTCCACTCCCTTA-3′; ACS FOR: 5-AGAGAGTAGTTATGGCTGGTGGTG-3′; ACS REV: 5′-GGAATGAGTTGTACTCCAGTTCTCC-3′. For semi-quantitative RT-PCR, 5 μg of RNA were converted to cDNA using M-MLV reverse transcriptase (Promega). One μl of each reverse transcriptase reaction was used as a template in a PCR reaction containing the specific primer pairs described above. 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. Analysis of the motion of endosomes prior to and following EIX treatment.

Figure S2. Endosomal mean square displacement (MSD) as a function of FYVE intensity after EIX treatment.

Movie S1. FYVE-DsRed endosomal marker localization in leaf tissue following EIX treatment.

Movie S2. FYVE-DsRed endosomal marker localization in leaf tissue.

Movie S3. FYVE-DsRed endosomal marker and GFP-LeEix2 localization in leaf tissue after EIX treatment.

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