Endocytosis of LeEix and EHD Proteins During Plant Defense Signalling

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Abstract Plants are exposed to pathogenic microorganisms in their environment, and have developed various defense mechanisms to avoid disease and death. Active defense reactions can also be triggered by treatment with microbial compounds called elicitors or microbial-associated molecular patterns (MAMPs), which may be characteristic of a whole group of organisms or limited to specific strains of a microbial species. Endocytosis has been demonstrated to be involved in plant immunity. EH domain-containing proteins (EHDs) are involved in various aspects of the endocytic process, primarily via protein–protein interactions. Here, we characterize endocytosis and signalling occurring during plant defense responses induced by elicitors, focusing particularly on EIX (ethylene inducing xylanase) and the involvement of EHD proteins in these processes.

1 Plant Defense Responses

The gene-for-gene (Flor 1947) model of plant–pathogen interactions proposes that each resistance gene (*R*-gene) confers resistance only to pathogens carrying the corresponding avirulence gene.

Gene-for-gene resistance responses have been observed in interaction of plants with a wide variety of pathogens, including fungi, bacteria, and viruses (Dewit

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et al. 1985; Blein et al. 1991; Fluhr et al. 1991; Basse et al. 1992). A simple molecular explanation for gene-for-gene resistance is that an avirulence gene encodes a ligand that binds to a receptor encoded by the plant *R*-gene (Ebel and Cosio 1994; Bent 1996). Ligand binding triggers activation of a signal transduction cascade culminating in expression of defense responses that inhibit the pathogen and confer the resistance (Glazebrook et al. 1997).

The defense mechanisms are triggered when an organic compound (termed elicitor) is recognized by a plant *R*-gene. The list of elicitors is long and includes glucans, pectic fragments, and proteins with and without carbohydrate side chains. Elicitors may be of pathogen or non-pathogen origin (Dewit et al. 1985; Blein et al. 1991; Basse et al. 1992; Furman-Matarasso et al. 1999).

Recognition between R-proteins and their corresponding elicitors is likely to activate a signal transduction cascade which involves various responses including cell wall fortification (Hammond-Kosack and Jones 1996), production of reactive oxygen species (ROS), induction of pathogenesis related (PR) genes (Hammond-Kosack and Jones 1996), Ethylene biosynthesis (Boller 1991), and the hypersensitive response (HR) (Yu et al. 1998; Elbaz et al. 2002).

In some cases, the "R" gene is a receptor which contains an endocytic motif, and endocytosis has been shown to be a crucial step in the recognition between the "R" gene and the elicitor in a few cases, including the LeEix system which will be described in detail below.

1.1 Plant Defense Receptors

Leucine-rich-repeat receptor kinase (LRR-RLKs) and LRR-RLPs are involved in signalling and 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 induction of defense responses (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).

LRR-RLPs have also been implicated in responses to pathogens. The tomato Cf genes which mediate resistance to *Cladosporium fulvum* encode LRR-RLPs, the LRR domain of which was shown to be important for avirulence (Avr) gene recognition (van der Hoorn et al. 2005). Additional LRR-RLPs include the tomato Ve-resistant proteins (Kawchuk et al. 2001; Fradin et al. 2009) and the LeEix proteins (Ron and Avni 2004).

*LeEix*1 and *LeEix*2 are responsible for the plant response to the fungal elicitor ethylene-inducing xylanase (EIX). The *LeEix* genes show homology to *R*-genes of transmembrane proteins which contain an extracellular LRR, like the *Cf* gene family and *Ve R*-genes. The LeEix proteins are transmembrane proteins and contain a signal

peptide within the N-terminus, an extracellular domain, a transmembrane domain, and a short cytoplasmic tail in the C-terminus. (Ron and Avni 2004).

1.2 Elicitors

Elicitors [microbial-associated molecular patterns (MAMPs)] that trigger plant defense responses have been isolated from a variety of phytopathogenic and non-pathogenic microorganisms (Fuchs et al. 1989; Ebel and Cosio 1994; Felix et al. 1999). In soybean cell culture, the *Verticillium* elicitor was shown to enter the cell via an endocytic process (Horn et al. 1989). Flg 22 stimulates endocytosis of FLS2, in a process which requires kinase activity (Robatzek et al. 2006). In tobacco, the cryptogein elicitor was reported to induce endocytosis in correlation with its defense response activation (Leborgne-Castel et al. 2008).

1.2.1 Ethylene-Inducing Xylanase

The plant hormone ethylene is involved in modulating a broad spectrum of physiological processes including pathogenesis, senescence, and fruit ripening (Goeschi et al. 1966).

A 22 kDa fungal β-1-4-endoxylanase protein referred to as EIX was isolated from xylan-induced *Trichoderma viride* cultures (Dean et al. 1989; Fuchs et al. 1989). Similar xylanases have been identified in xylan-induced filtrates of plant pathogenic fungi (Dean et al. 1989; Wu et al. 1997). Injection of EIX into the leaf mesophyll intercellular spaces induces ethylene production and HR, as well as other plant defense responses in *Nicotiana tabacum* cv. Xanthi (Fuchs et al. 1989; Lotan and Fluhr 1990; Bailey et al. 1990, 1992; Dean and Anderson 1991), *Solanum lycopersicon* (tomato) leaf tissue (Ron et al. 2000), *Nicotiana tabacum* cell suspensions (Yano et al. 1998), and in other plant species. These responses are characteristic of plants responding to exogenously applied elicitors (Blein et al. 1991; Felix et al. 1993).

EIX induces defense responses in specific plant species and/or varieties (Bailey et al. 1990, 1992; Ron et al. 2000; Elbaz et al. 2002), and was shown to specifically bind to the plasma membrane of both tomato and tobacco EIX-responding cultivars (Hanania and Avni 1997).

2 Endocytosis in Plant Defense Responses

Many apparent roles for regulated endocytosis in plant development and immunity have emerged (Robatzek 2007). The tomato Ve2, Cf9, Cf4, and LeEix 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. However, the extensively studied flagellin defense response receptor FLS2 does not contain a Yxxϕ motif, but it was reported to contain a PEST-like motif which has also been implicated in endocytosis (Robatzek et al. 2006). Mutation in the endocytic motif of LeEix2 resulted in abolishment of HR induction in response to EIX, suggesting 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 (TLR), which also contain extra-cellular LRR domains (Husebye et al. 2006).

3 Epidermal Growth Factor Receptor Substrate-15 (EPS-15) Homology Domain Containing Proteins in Plant Cells

Endocytosis involves many protein–protein interactions. One module which mediates such interactions is the EH domain-containing protein (EHD) (EPS15 homology domain) first identified in EPS15 (Wong et al. 1995; Carbone et al. 1997). Sequence analysis and preliminary functional characterization of plant EHDs suggest a high level of functional homology with mammalian EHDs. Orthologs of EHD proteins exist in Arabidopsis and the entire *Solanaceae* family as well as in rice, maize, and other plant species. The Arabidopsis EHD proteins share high homology with their mammalian counterparts (Mintz et al. 1999; Pohl et al. 2000; Galperin et al. 2002). Structurally, AtEHD1, AtEHD2, and its spliced variant termed AtEHD2-2 contain the same domains as the mammalian proteins (Pohl et al. 2000; Galperin et al. 2002; Naslavsky and Caplan 2005). The EH domain is present at the N-terminus of the plant proteins, with the center domain harboring the nucleotide binding site and the DxxG motif that is completely conserved in evolution (Rotem-Yehudar et al. 2001; Galperin et al. 2002). A major difference between the plant EHD proteins and the mammalian, Drosophila, and *Caenorhabditis elegans* proteins is the location of the EH domain. While many N-terminal EH domain-containing proteins exist in mammals and other organisms (Naslavsky and Caplan 2005), the AtEHDs described herein bear the most resemblance to the mammalian EHDs (EHD1-4). The conservation of EHD proteins throughout the plant kingdom and their relatively high homology to the mammalian EHDs indicate their relative importance in plant systems. Despite the difference in domain arrangement, we have demonstrated that the plant EHD proteins have similar functions as their mammalian counterparts. Available microarray data (Zimmermann et al. 2004) indicate that the Arabidopsis *EHD* genes are expressed in all plant tissues. This correlates with the data obtained for the mammalian EHD proteins (Mintz et al. 1999; Pohl et al. 2000; Rapaport et al. 2006; George et al. 2007), and may also indicate the importance of EHDs in

ubiquitous functions occurring in all types of cells. Given the role of EHDs in endocytosis, it is likely that these conserved proteins serve integral roles in signalling in a variety of cell types in diverse species (Polo et al. 2003).

AtEHD1 and AtEHD2 are localized to endosomes and colocalize with endocytic markers in both plant and mammalian systems (Bar et al. 2008). The fact that both proteins colocalize with FM4-64 shows that they are localized to membranous endocytic organelles.

AtEHD1 fully colocalizes with hEHD1 and hEHD3 but does not colocalize with hEHD2 or hEHD4 (Bar et al. 2008). This could indicate that AtEHD1 and hEHD1/3 share similar functions, which is consistent with reported phenotypes for hEHD1 in knock-out mice.

Arabidopsis plants silenced in the *AtEHD*1 gene demonstrated a delay in internalization of the fluorescent dye FM4-64 (Bar et al. 2008). As AtEHD1 colocalizes with hEHD1, it is possible that this delay may be a similar phenomenon to the delayed recycling observed in hEHD1 knock-out mice (Rapaport et al. 2006). Silenced *AtEHD*1 plants did not show any distinctive phenotype. By contrast, *AtEHD*2 overexpression suppresses endocytosis in both plant and mammalian cells (Bar et al. 2008), as does hEHD2 in mammalian cells (Guilherme et al. 2004a). Thus, it is possible that AtEHD1 and AtEHD2 have coevolved in plants to exert opposite effects; one may act to stimulate endocytosis under certain conditions, while the other one can suppress endocytosis under certain conditions. The rate of endocytosis depends on a multitude of factors, and many of them remain unknown. However, these parameters could be influenced by the expression level (or other regulatory elements) of one or both AtEHDs. One could envisage a decrease of active AtEHD1 or an increase of active AtEHD2 (or vice versa) in a situation when endocytosis must be precisely regulated.

Considering the inhibitory effect of AtEHD2 overxpression on endocytosis, it is possible that AtEHD2 is involved in a particular rate-limiting step of the endocytic process. In such case, overexpression of AtEHD2 may cause the endocytic process to become "stuck" in this particular step, thus inhibiting faster entry of typically endocytosed material into the cell. This could also explain why the expression level of *AtEHD*2 is normally very low in wild-type cells compared to the expression of *AtEHD*1 (Bar et al. 2008).

3.1 EHD1 and Recycling

We have recently demonstrated that knock-down of *EHD*1 causes a delayed recycling phenotype and reduces brefeldin A sensitivity in Arabidopsis seedlings (Bar and Avni, unpublished results). Interestingly, internalization of LeEix2 depends primarily on recycling as opposed to de novo protein synthesis (Bar and Avni 2009a). The EH domain of EHD1 was found to be crucial for the localization of EHD1 to endosomal structures. Mutant EHD1 lacking the EH domain did not localize to endosomes and showed a phenotype similar to that of *EHD*1

knock-down seedlings. Mutants lacking the coiled-coil domain, however, showed a phenotype similar to wild-type or *EHD*1 overexpressing seedlings. Interestingly, transgenic plants overexpressing *EHD*1 possess enhanced tolerance to salt stress, a property which also requires an intact EH domain.

3.2 EHD2 and Inhibition of Endocytosis

Notably, mammalian EHD2 appears to be the most unique mammalian EHD protein, and the same is true for plant EHD2 (Bar et al. 2008). This is interesting, given that EHD2 is not usually endosomal and it was the only EHD protein found to inhibit endocytosis both in mammals and Arabidopsis.

AtEHD2 does not significantly colocalize with any of the mammalian EHD proteins (Bar et al. 2008). Though plant proteins are by no means guaranteed to localize properly in mammalian cells, it would seem that AtEHD2 shares similar function with hEHD2 based on the inhibitory effect on endocytosis. This effect was observed both in plant cells and in mammalian cells using plant AtEHD2, indicating that AtEHD2 is able to exert at least some of its native biological activity in mammalian cells. This shows the high level of functional homology between plant and mammalian endocytosis (Ortiz-Zapater et al. 2006; Lam et al. 2007a).

AtEHD1 was found to be colocalized with ARA6 and FYVE (Bar et al. 2008) as well as with RabA1e and RabD2b (Bar and Avni, unpublished results), indicating that it resides partly on early endosomes (Ueda et al. 2001; Ŝamaj et al. 2005; Voigt et al. 2005; Golomb et al. 2008), from which recycling back to the plasma membrane occurs in plant cells (Ueda et al. 2001). Mammalian EHD1 was found to reside primarily in the endocytic recycling compartment (ERC) (Mintz et al. 1999; Grant et al. 2001), as well as on early endosomes (Naslavsky et al. 2004) and vesicular/tubular structures (Caplan et al. 2002). Though evidence of recycling endosomes exists in plants (Jaillais and Gaude 2007; Jaillais et al. 2008), such endosomes have not been well characterized. Previous work in the field of plant endocytosis has shown that materials are recycled to the TGN and back to the plasma membrane from early endosomes (Lam et al. 2007a, b). Thus, AtEHD1 resides on early endosomes and recycling endosomes, which may partially overlap. From EHD1 positive endosomes, some of the PM receptors are recycled back to the cell surface. Mammalian and plant EHD1 clearly function together with other endocytic/recycling proteins, as knock-down of *EHD*1 results only in a mild recycling phenotype, in both plants and mammals (Rapaport et al. 2006; Bar et al. 2008).

EHD2 resides primarily at the plasma membrane. Though the expression level of *AtEHD*2 is very low under normal conditions, upon overexpression it acts to diminish internalization of such "classical" endocytic cargos as FM4-64 and transferrin in plant and mammalian cells, respectively (Guilherme et al. 2004a; Bar et al. 2008). EHD2 most likely inhibits the clathrin-dependent endocytic pathway (Dhonukshe et al. 2007; Bar et al. 2008), though it could possibly affect other pathways as well.

Plant EHD2 inhibits endocytosis of other receptors upon overexpression, including LRR-receptor-like proteins in plant cells (Bar and Avni 2009a, b). We have previously shown (Ron and Avni 2004) that signalling of the tomato LeEix2 receptor requires the endocytic process. EHD2 controls LeEix2 signalling via modulation of its endocytosis, thereby limiting the level of the plant response. Plant EHD2 may also serve to modulate other signalling processes in which endocytosis is involved. For example, plant EHD2 may regulate auxin signalling via regulation of PIN (auxin efflux transporter) endocytosis which is clathrin dependent (Dhonukshe et al. 2007). This could indicate that plant EHD2 is part of a more general ubiquitous control mechanism associated with receptor-mediated endocytosis (RME, see also Chap. 7 by Di Rubbo and Russinova in this volume).

Both EHD1 and EHD2 were found to be coupled to the actin cytoskeleton in mammals (Guilherme et al. 2004a; Braun et al. 2005). Our results demonstrate that this is the case in plants as well (Bar et al. 2008, 2009). It is not clear at this point how EHD2 exerts its function in RME under native conditions, but one clue could be that it shows plasma membrane and not endosomal localization in both mammalian (Benjamin et al. 2011) and plant cells (Guilherme et al. 2004a; Bar et al. 2008). Perhaps fluctuations in local concentration of EHD2 at micro domains within the plasma membrane can regulate the level of endocytosis at different locations throughout the cell. Soluble TLR was shown to inhibit the signalling of membrane TLR by binding to the TLR-specific ligand, thus serving as decay receptors. Similarly, the expression level of *EHD*2 may modulate the endocytic process and provide negative regulation when required.

EHD2 appears to be an essential component in the endocytosis of the LeEix2, Cf4, and Cf9 receptors, causing inhibition of HR and ethylene biosynthesis upon its overexpression, while it does not seem to be involved in the FLS2 system (Bar and Avni 2009a, b). EHD2 may affect endocytosis directly, though it is also possible that EHD2 modulates LeEix2 internalization through an effect on the plasma membrane.

3.3 Functional Analysis of EHD2 Domains

We conducted an analysis of the importance of various domains within EHD2 for the protein function. The coiled-coil domain of EHD2 is crucial for the ability of EHD2 to inhibit endocytosis in plants (Bar et al. 2009). This domain was also required for binding of EHD2 to the LeEix2 receptor. Therefore, we suggest that binding of EHD2 to the LeEix2 receptor is required for inhibition of LeEix2 internalization. The P-loop of EHD2 is important for EHD2 to function properly (Bar et al. 2009), as evidenced by the loss of the ability to inhibit EIX-induced HR and to bind LeEix2 in the *AtEHD*2_*G*221*R P-loop* mutant. Our observations together with the published importance of the P-loop in mammalian EHDs suggest the possibility that the P-loop is required for proper membrane localization of AtEHD2, while the coiled-coil in fact mediates the binding to "target" proteins,

thereby enabling the inhibitory function on endocytosis. Neither the P-loop mutant (G221R) nor a coiled-coil deletion (ΔCC) was able to bind the LeEix2 receptor, and both mutants lost the ability to inhibit HR.

Interestingly, the EH domain of AtEHD2 does not appear to be involved in the inhibition of endocytosis. Both a point mutation in the EH domain (G37R) and a complete deletion of this domain (ΔEH) did not affect the inhibition of endocytosis, as these mutants retained wild-type level activity. Further, swapping the EH domain between AtEHD1 (which does not inhibit endocytosis) and AtEHD2 had no effect. As mentioned above, EHD2 is localized primarily to the plasma membrane in both mammals and plants. Interestingly, a truncation mutant of mammalian EHD2 lacking the EH domain was also localized to the plasma membrane (Blume et al. 2007). Additionally, this mutant was able to inhibit internalization of transferrin in a manner similar to that of wild-type EHD2 (Guilherme et al. 2004a, b). It seems that although the EHDs share a high level of homology and similar structure/domains, in mammals and plants, the fact that each EHD possesses different functions could be related to the different domains present in the protein, whereby each function is exerted primarily through a different domain. Thus, different domains might have varying importance in different EHD proteins. The EH domain, which appears to be very important in EHD1, may not be crucial for the function of EHD2.

We suggest that upon EIX binding, μ-adaptin binds to the YXXϕ motif within the cytoplasmic domain of the LeEix2 receptor. The AP-2 complex is assembled, and AtEHD2 binds the ó-subunit of AP-2 and/or the LeEix2 receptor directly via the coiled-coil domain (Bar et al. 2009). However, the involvement of additional proteins in this complex cannot be excluded. Tethering of this complex to the actin cytoskeleton via additional proteins, as was reported for EHD2 in mammals (Guilherme et al. 2004a), may take part in the inhibition of endocytosis, particularly given the actin reorganization phenotype caused by *EHD2* overexpression (Bar et al. 2009). The binding of AtEHD2 to AP-2 and/or LeEix2 needs to be examined further in order to elucidate the activity of different protein complexes in LeEix2 internalization and function.

4 LeEix2/EIX Endocytosis

4.1 Parameters of LeEix2 Endocytosis

Endocytosis is a crucial step in the defense response triggered in plants by EIX and additional MAMPs. Similar to previous work done with FLS2 (Robatzek et al. 2006), we were able to show (Bar and Avni 2009b) that signalling of the fungal elicitor EIX is dependent on internalization of its receptor LeEix2 via endocytosis, in a process which requires components of the cytoskeleton. After EIX application (15–20 min), LeEix2 is internalized into highly motile endosomes, in a swift endocytic process which follows a similar time course to that described for

flg22-induced FLS2 (Robatzek et al. 2006), and similar to the time-frame of mammalian endocytosis (Gruenberg and Howell 1987).

LeEix2, Cf9, and Cf4 are LRR-RLPs showing structural similarities. They all possess extra-cellular LRR repeats and short cytoplasmic domains containing the Yxxϕ endocytic motif (Jones et al. 1994; Ron and Avni 2004). FLS2 is a receptor-like kinase (LRR-RLK) and has an intra-cellular kinase domain which does not contain theYxxϕ motif but instead it contains a non-classical PEST-like endocytic motif (Gomez-Gomez and Boller 2000; Robatzek et al. 2006). Another difference between LeEix2 and FLS2 is that FLS2 appears to be degraded and synthesized de novo after flg22-induced internalization, while LeEix2 is probably returned (at least in part) to the plasma membrane by recycling vesicles, given that cycloheximide does not affect its presence in the membrane (Bar and Avni 2009b). The recycling of LeEix2 does not require protein synthesis but may be amplified by the synthesis of certain proteins involved.

Further evidence that EHD2 is specific to the endocytic pathway of LeEix2 but not FLS2 comes from the fact that EIX but not flg22 can induce the expression of *NtEHD2* (Bar and Avni 2009b). Thus, EIX application triggers *NtEHD2* expression, and NtEHD2 acts to inhibit the defense response in the short term. Longer exposure to the MAMP leads to a "full-blown" defense response including HR which is free of the EHD2 inhibitory influence, suggesting that a control mechanism based on the interplay of different proteins may be at work. The kinase activity of RLKs such as FLS2 may be required for receptor internalization and signalling (Robatzek et al. 2006) and may provide the specificity which is not possible in the case of LeEix2 or Cf9. Concerning LeEix2 and Cf9, one could envisage a mechanism in which the MAMP triggers expression of the endocytosis inhibitory protein in order to more tightly regulate the HR.

4.2 Characterization of EIX Endocytosis

Internalization of defense receptors is required for proper defense signalling in several cases (Ron and Avni 2004; Robatzek et al. 2006; Bar and Avni 2009a). As we have previously reported, internalization of both EIX (Hanania and Avni 1997; Rotblat et al. 2002) and the LeEix2 receptor (Ron and Avni 2004; Bar and Avni 2009a) are required for the plant to mount a proper response to EIX. Prevention of internalization of endocytic vesicles by inhibition of dynamin further demonstrated the necessity of EIX/LeEix2 internalization for signalling and defense responses. The same is true for inhibition of the actin cytoskeleton using the F-actin depolymerizing drug latrunculinB (Sharfman et al. 2011).

Characterization of endosomal movement and content following EIX treatment allowed us to demonstrate that EIX causes a sub-population of endosomes to move faster and in a more directional manner (Sharfman et al. 2011). Further, this subpopulation appears to contain smaller endosomes or endosomes with a lower PI-3-P content (as revealed by FYVE, a PI-3-P binding molecular marker) (Voigt

et al. 2005; Sharfman et al. 2011). We have also shown that at least some of the endosomes containing LeEix show increased speed and directionality of movement (Sharfman et al. 2011). Given the requirement of an intact cytoskeleton for EIX signalling, it seems probable that directional movement following EIX treatment occurs on actin filaments.

In addition, components of membrane lipid synthesis and signalling are involved in the plant response to EIX. Thus, manipulating PLD or PLC activity impairs membrane functions and ultimately leads to inability of the cell to form endosomes, which in turn prevents endocytosis. Moreover, certain products of the membrane lipid synthesis such as phosphatidic acid may themselves serve as secondary messengers and be involved to a certain extent in EIX/LeEix2 signalling.

In addition to endocytosis of both the receptor and elicitor, proper EIX signalling also requires tyrosine kinase activity (Sharfman et al. 2011). The LeEix2 receptor is devoid of kinase activity, unlike some plant defense receptors such as FLS2 (Gomez-Gomez and Boller 2000; Robatzek et al. 2006), but may be phosphorylated by another protein. If the LeEix2 receptor is phosphorylated, such phosphorylation may be required for receptor internalization and for proper signalling. Alternatively, tyrosine kinase activity may be involved in further downstream signalling events ensuring proper plant response to EIX.

5 Conclusions and Future Prospects

Results obtained in connection with LeEix2 internalization indicate that recycling might be involved in some aspects of EIX signalling, as inhibition of protein synthesis did not result in receptor degradation following internalization, but did cause LeEix2 to persist on endosomes (Bar and Avni 2009a). Additional results suggest that recycling of the EIX receptor is perhaps responsible for the amplitude of the response to EIX, since treatment of cells with BFA caused attenuation of EIX signalling in several examined parameters (Sharfman et al. 2011).

signalling endosomes have been previously reported in plants (Geldner et al. 2007). Considering data obtained upon temporal separation of the internalization event (by several inhibitors) from the endosome trafficking (by treatment with endosidin1 (Robert et al. 2008), it seems that much of the EIX-LeEix2 signalling occurs from endosomal compartments (Sharfman et al. 2011). Further exploration of endosomal signalling in the EIX/LeEix2 system as well as in plants in general is required.

Endocytosis is involved in both biotic and abiotic stresses in plant cells and the EHD proteins as regulators of endocytosis participate in plant responses to these stresses. The elucidation of various components of EIX/LeEix2 endocytosis and signalling provides framework for future research aiming to further clarify plant defense signalling mechanisms. Based on previous and recent work presented here (Hanania et al. 1999; Rotblat et al. 2002; Ron and Avni 2004; Bar et al. 2008), we propose the model depicted in Fig. 1.

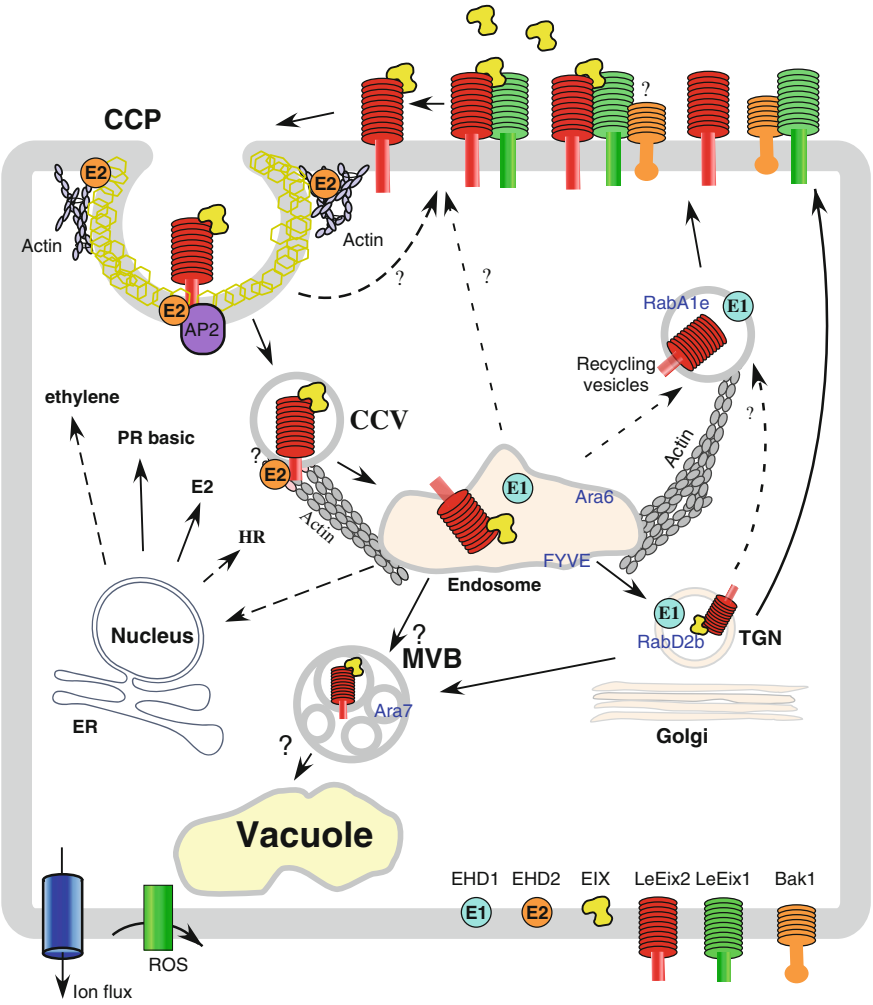

Fig. 1 Proposed model for EIX-mediated LeEix endocytosis and signalling. Upon EIX application, it binds the LeEix2 receptor on the outside of the plasma membrane (Hanania and Avni 1997; Ron and Avni 2004). The expression of both LeEix receptors causes attenuation of EIX endocytosis and signalling, in a BAK1 dependent manner (Bar et al. 2011). The EIX-LeEix2 complex probably binds an endocytic protein complex using the Yxxϕ motif present within the cytoplasmic tail of LeEix2. Two subunits of AP-2 bind to the Yxxϕ motif of LeEix2 and to EHD2, respectively (Bar and Avni 2009a; Bar et al. 2009). LeEix2 and EIX are internalized into FYVE endosomes, which probably also contain EHD1 (Bar et al. 2008). LeEix2 is probably recycled back to the plasma membrane on recycling vesicles which contain RabA1e and also EHD1, as well as TGN/early endosomes, which may overlap with recycling vesicles, and contain RabD2b and EHD1 (Bar and Avni, unpublished results) The internalization of LeEix2 and EIX is required for induction of defense responses, (Bailey et al. 1990, 1992; Laxalt et al. 2007). EIX application also triggers EHD2 and LeEix1 expression, which act to inhibit the defense response in the short term. Longer exposure to EIX (or other elicitors) leads to a "full-blown" defense response. LeEix2 and possibly also EIX are likely also partially degraded via the multivesicular body (MVB) pathway

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