ENDOCYTOSIS IN PLANT – FUNGAL INTERACTIONS

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1. Introduction

Plants are continuously exposed to pathogenic microorganisms in their environment, and possess many mechanisms aimed at mounting an effective defense against these pathogens (Jones and Dangl, 2006; Yang et al., 1997). These defense responses include the strengthening of mechanical barriers, oxidative burst, "*de novo*" production of antimicrobial compounds such as pathogenesis-related (PR) proteins and phytoalexins, and the induction of the hypersensitive response (HR) mechanism, where the tissue surrounding the infection site dies and confines pathogen growth (Hammond-Kosack and Jones, 1996).

The host plant recognizes foreign molecules associated with microorganisms. Some recognition events conform to the model in which a host receptor interacts directly with a molecule of the microbe. These include the interaction between microbe-associated molecular patterns (MAMPs) and MAMP receptors (Nurnberger et al., 2004). They also include the interaction between some effectors and their cognate resistance (R) proteins. Elicitors (MAMPs) that trigger plant defense responses have been isolated from a variety of phytopathogenic and nonpathogenic microorganisms (Ebel and Cosio, 1994; Felix et al., 1999; Fuchs et al., 1989; Ricci et al., 1993) The ability of the plant to recognize and defend itself upon MAMP perception has recently been studied extensively in the context of endocytosis, in particular in connection with bacterial proteins (Martin et al., 2003; Robatzek et al., 2007). Here we present an analysis relating primarily to fungal MAMPs

1.1. VERTICILLIUM GLYCOPROTEINS

Fungi of the genus *Verticillium* are pathogens responsible for vascular wilt disease in over 200 plant species (Fradin and Thomma, 2006). A few elicitors present in *Verticillium* species have been previously documented, among them a 65 kDa heat-stable glycoprotein (Davis et al., 1998). A locus responsible for resistance against *Verticillium*, termed *Ve*, has been isolated from tomato and was found to confer resistance to strains of *V. dahliae* and *V. alboatrum*. The *Ve* locus contains two genes: *Ve1* and *Ve2*, which encode cell surface leucine-rich repeat (LRR) receptor-like protein (LRR-RLPs; Kawchuk et al., 2001).

1.2. CRYPTOGEIN FROM *PHYTOPHTHORA*

Cryptogein and Capsicein are proteinaceous elicitors isolated from the oomycete *Phytophthora* (water mold) and are capable of eliciting defense responses in tobacco; Cryptogein is 50 times more potent than Capsicein (Ricci et al., 1989).

Cryptogein induces hypersensitive response (HR) and systemic acquired resistance (SAR) in tobacco plants (Lebrun-Garcia et al., 1999). The tobacco response to cryptogein also includes production of active oxygen species, cytosol acidification, membrane depolarization, and MAP kinase activation (Lebrun-Garcia et al., 1999).

Cryptogein was found to bind tobacco plasma membranes in a saturable, specific, and reversible manner, in concentrations required for in vivo activity. The putative Cryptogein receptor may also be glycosylated (Wendehenne et al., 1995).

1.3. *CLADOSPORIUM FULVUM* AVR PROTEINS

C. fulvum causes leaf mold disease on sensitive cultivars of Tomato. Tomato *Cf* genes confer resistance to *C. fulvum* through recognition of fungal Avr proteins. Many tomato *Cf* genes have been cloned. The encoded proteins are type I transmembrane glycoproteins containing extracellular leucine-rich repeats (LRRs), a membrane spanning region and a short cytoplasmic domain (Rivas and Thomas, 2005).

Many host responses have been described as characterizing the interaction between Cf and corresponding Avr proteins, including deposition of callose, production of glucanases and chitinases, production of phytoalexins and pathogenesis-related (PR) proteins, as well as production of active oxygen species, stimulation of protein kinases, and hypersensitive response (HR; Joosten et al., 2000; Joosten and de Wit, 1999). However, no physical interaction was detected between Cf4 or Cf9 and their corresponding Avr proteins, though the possibility was examined in many different experimental systems (Luderer et al., 2001; Rivas and Thomas, 2005). In fact, though Avr4 and Avr9 are the presumed ligands of Cf4 and Cf9, respectively, the molecular mechanism underlying Avr protein perception has not been established.

Interestingly, and possibly due in part to the lack of direct physical interaction between the Cf and Avr proteins, though the tomato Cf receptors Cf4 and Cf9 were reported to contain the conserved endocytosis signal Yxxɸ within the short cytoplasmic domain (Jones et al., 1994b; Thomas et al., 1997), endocytosis of the corresponding Avr proteins has not been reported. However, vesicular transport and signaling are no doubt involved in the response to *C. fulvum* Avr proteins, as evidenced by the specific phosphorylation of a syntaxin (SNARE complex protein) early in the Cf9/Avr9 pathway (Heese et al., 2005).

[AU1]

1.4. ETHYLENE-INDUCING XYLANASE FROM *TRICHODERMA*

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 (Avni et al., 1994; Bailey et al., 1990). EIX induces ethylene biosynthesis, electrolyte leakage, expression of PR proteins, and HR in specific plant species and/or varieties (Bailey et al., 1990; 1992; Elbaz et al., 2002; Ron et al., 2000). EIX was shown to specifically bind 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 receptor-like-protein (LRR-RLP) encoded by a single dominant locus, termed LeEix (Ron and Avni, 2004).

2. Endocytosis in Plants

In the past years, the roles of regulated endocytosis in plant development and plant immunity are emerging (Robatzek, 2007). A variety of membranal receptors, mostly leucine-rich repeat (LRR) receptors, have been identified and are involved in many processes, including cell differentiation and defense signaling. Ligand-dependent (Robatzek et al., 2006) and ligand-independent, constitutive receptor internalization have been documented (Gifford et al., 2005; Shah et al., 2002). As is the case for cell surface receptors in mammalian cells, autophosphorylation of the cytosolic domain of plant LRR-RLK receptors induced by ligand binding has also been demonstrated (Shah et al., 2002). Plant receptors can also undergo recycling back to the PM after internalization (Albrecht et al., 2008). However, despite many recent advances in the field, plant endocytic compartments are not well characterized and the term endosome is often employed generally for compartments containing endocytosed material. 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 (Gross et al., 2005; Lam et al., 2007b; Muller et al., 2007; Samaj et al., 2004, 2005).

Styrl dyes such as FM-4-64 have been used to study localization of vesicles, which are putative endosomes (Bolte et al., 2004; Grebe et al., 2003; Lam et al., 2007a; Ueda et al., 2001). Structural studies indicated that the partially coated reticulum (PCR) is analogous to the early/recycling endosomes of mammalians (Galway et al., 1993). Two distinct classes of early endosomes were identified in *Arabidopsis*. One comprises the endosomes in which Ara6 resides, and the other the endosomes to which Ara6 is not targeted (Ueda et al., 2001). Early endosomes have also recently been characterized as SCAMP1 containing tubular-vesicular structures possessing clathrin coats and residing in the vicinity of the trans-golgi (Lam et al., 2007b). Molecule sorting occurs in the early endosomes, from which they are either recycled back to the plasma membrane, transported to the golgi apparatus, or to multivesicular bodies (MVBs, also known as late endosomes; Battey et al., 1999; Jurgens, 2004). The trans-golgi network (TGN) was also found to be involved in early endocytic pathways in Arabidopsis (Dettmer et al., 2006; Lam et al., 2007b). Prevacuolar compartments (PVCs) have been identified as

117 MVBs in tobacco BY2 cells (Tse et al., 2004). From the MVBs, the endocytosed
118 material is targeted to the vacuole for degradation. Recent studies conducted in
119 plant systems have further elucidated possible functionalities of plant endocytic
120 compartments and the flow of endocytosed material throughout plant cells
121 (Geldner and Robatzek, 2008; Lam et al., 2007a; Muller et al., 2007; Silady et al.,
122 2008; Teh and Moore, 2007).
123   Clathrin-coated vesicles are most probably a major means of internalization in
124 plant cells. Studies conducted recently have demonstrated that clathrin-dependent
125 internalization occurs in plants (Dhonukshe et al., 2007; Lam et al., 2007b;
126 Leborgne-Castel et al., 2008; Perez-Gomez and Moore, 2007; Tahara et al., 2007).
127 Components that interact with the clathrin-coated vesicles and adaptor proteins
128 such as dynamins and proteins that contain an SH3 domain occur in plants and are
129 involved in endocytosis and vesicle trafficking (Kang et al., 2003; Lam et al., 2001).

130 **3. Ligand-Induced Endocytosis of Fungal Elicitors**

131 Fungal elicitors have been shown to enter plant cells in several instances. In some
132 cases, a specific plant receptor, which recognizes the fungal elicitor, has been identi-
133 fied and isolated. Thus far, many of the plant receptors identified, which recognize
134 fungal elicitors have been shown to be leucine-rich-repeat receptor-like proteins
135 (LRR-RLPs), which contain an extracellular LRR and lack a kinase domain.
136 LRR motifs are often found in proteins involved in specific protein–protein inter-
137 actions. In the case of some R proteins, the LRR domains are believed to deter-
138 mine the specificity of Avr ligand binding (Hammond-Kosack and Jones, 1997;
139 Thomas et al., 1997). Additionally, some of these receptors contain a Yxxϕ motif
140 for clathrin-mediated endocytosis.
141   One of the first systems, which demonstrated that endocytosis does indeed
142 occur in turgid plant cells employed the *Verticillium* elicitor. Although this work
143 dates to 1989 it is still current today, and it was the first to indicate the possibility
144 of receptor-mediated endocytosis in plant cells. This work indicated that there
145 probably exists a specific receptor for the *Verticilium* elicitor (Horn et al., 1989).
146 Subsequently, two *Ve* receptors were isolated, and found to be LRR-RLPs con-
147 taining the Yxxϕ endocytosis motif (Kawchuk et al., 2001) as detailed above.
148   The *Verticillium* elicitor was shown to enter the cell by an endocytic process
149 in soybean cell cultures (Horn et al., 1989). The rate of elicitor uptake as well as
150 its sensitivity to temperature conform with an endocytic process. A preparation
151 of the *Verticillium* elicitor, which is a glycoprotein as described above, was found
152 to associate with the cell surface and subsequently induce the formation of H_2O_2
153 within 5 min. Internalization of the labeled elicitor was competitively inhibited by
154 unlabeled elicitor and, 5–7 h after application, a large portion of the elicitor was
155 delivered to the cell vacuole, probably for degradation (Horn et al., 1989).
156   The Cryptogein elicitor was recently shown to induce endocytosis in correla-
157 tion with its defense response activation (Leborgne-Castel et al., 2008). Endocytosis

of the lipophylic dye FM-4-64, which is commonly accepted as a marker for clathrin-mediated endocytosis in plant, was found to be stimulated in response to the addition of Cryptogein to a tobacco cell suspension. However, endocytosis of FM-4-64 was not induced in response to a control ligand, which does not trigger defense response signaling. Additionally, cryptogein was found to induce a transitory stimulation of clathrin-coated pits within 15 min of its addition. Both these phenomena were blocked in the presence of tyrphostin A23, which can inhibit receptor-mediated endocytosis. The study presented in Leborgne-Castel et al. (2008) is one of the first to link clathrin-coated pits and vesicles with the endocytosis of a plant defense response elicitor and, given the evidence presented, most probably occurs via a specific receptor, which may contain a clathrin-mediated endocytosis motif.

Hanania et al. (1999) showed that after binding the plant membrane EIX is transported into the cytoplasm. Mutation in the endocytosis motif of LeEix2 resulted in abolishment of induction in HR in response to EIX, suggesting that endocytosis plays a key role in mediating the signal generated by EIX that leads to HR induction (Ron and Avni, 2004).

[AU2] In a recent work (M. Bar and A. Avni, unpublished results), we have shown that EIX triggers internalization of the LeEix2 receptor on endosomes, which are dependent on an intact cytoskeleton. Ten to 15 min after EIX application the GFP-tagged LeEix2 receptor can be seen throughout the cell on vesicles. These vesicles were also FYVE positive indicating that they are endosomes. In untreated leaves, GFP-tagged LeEix2 did not appear colocalized with the FYVE marker (data not shown). The FYVE domain has been reported to localize to endosomes in mammalian cells (Stenmark et al., 1996) as well as plant cells (Heras and Drobak, 2002; Jensen et al., 2001; Voigt et al., 2005). The FYVE-positive LeEix2 vesicles were also highly motile, as is characteristic of endosomes. In untreated leaves the FYVE vesicles have similar motility, while the GFP-LeEix2 is localized to the plasma membrane.

4. Defense Receptors and the Involvement of the Endocytic Mechanism in Plant Defense Response Signaling

Leucine-rich-repeat receptor kinase (LRR-RLKs) and LRR-RLPs have been implicated in signaling as well as defense responses in plants (Becraft, 2002; Torii, 2004). 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 and Boller, 2000; Gomez-Gomez et al., 1999). FLS2 is responsible for flagellin recognition, leading to a response, which includes generation of ROS, MAP kinase activation, ethylene production, and induction of gene transcription (Asai et al., 2002; Felix et al., 1999; Zipfel et al., 2004). The perception of flagellin by FLS2 was shown to be essential for the plant defense response, as FLS2 mutations compromised the ability of the plant

199 to mount an efficient defense against bacterial pathogens (Robatzek et al., 2006;
200 Zipfel et al., 2004). Interestingly, the kinase activity of RLKs such as FLS2 may
201 be required for receptor internalization and is probably required for receptor sig-
202 naling (Robatzek et al., 2006).

203 As detailed above, LRR-RLPs have been implicated in response to patho-
204 gens. The tomato *Cf* genes, which mediate resistance to *C. fulvum* encode LRR-
205 RLPs, the LRR domain of which was shown to be important for avirulence (Avr)
206 gene recognition (Takken et al., 1999; van der Hoorn et al., 2005). Genetic compat-
207 ibility of a Cf protein and its Avr counterpart typically leads to defense responses
208 including oxidative bust, ion fluxes, MAP kinase activation, and induction of HR
209 (May et al., 1996; Piedras et al., 1998; Romeis et al., 1999) that inhibits *C. fulvum*
210 proliferation. Additional LRR-RLPs include the tomato *Ve*-resistant proteins
211 (Kawchuk et al., 2001) and the *LeEix* proteins, as mentioned above (Ron and Avni,
212 2004). The tomato Ve2-, Cf9-, Cf4-, and LeEix-resistant proteins detailed herein
213 (Jones et al., 1994; Kawchuk et al., 2001; Ron and Avni, 2004; Takken et al., 1998)
214 contain the conserved endocytosis signal Yxxϕ within the short cytoplasmic
215 domain. Mutating this signal in LeEix2 abolishes both endocytosis (M. Bar and [AU3]
216 A. Avni, unpublished) and receptor signaling (Ron and Avni, 2004).

217 ## 4.1. THE ENDOCYTIC MECHANISM INVOLVED IN PLANT
218 DEFENSE RESPONSES TRIGGERED BY FUNGAL
219 ELICITORS: EIX AS A MODEL

220 A schematic proposed model incorporating our works relating to the LeEix recep-
221 tor (Bar et al., 2008; Hanania et al., 1999; Ron and Avni, 2004; Rotblat et al.,
222 2002) is presented in Fig. 1. Upon EIX application, EIX binds the LeEix2 recep-
223 tor on the outside of the plasma membrane (Hanania and Avni, 1997; Ron and
224 Avni, 2004). This binding was shown not to require additional plant proteins (Ron
225 and Avni, 2004). The ligand–receptor complex probably signals for the binding of
226 an endocytic protein complex to the Yxxϕ motif present within the cytoplasmic
227 tail of LeEix2. One protein in such a complex could be AP-2, which has been
228 shown to bind the Yxxϕ motif of transferrin receptor and participates in transfer-
229 rin internalization in *Arabidopsis* protoplasts (Ortiz-Zapater et al., 2006). Binding
230 of AP-2, usually via the Yxxϕ motif, has also been shown to be a crucial step in
231 the internalization of several mammalian receptors (Traub, 2003).

232 Interestingly, EHD proteins in mammalians were shown to bind adaptor
233 proteins, as well as additional proteins of the clathrin-coated vesicle complex
234 (Rotem-Yehudar et al., 2001). This may also be the case in plant cells. It is pos-
235 sible that AP-2 resides in a complex with the cytoplasmic tail of LeEix2 and
236 EHD2, as well as additional proteins.

237 Binding of EIX allows for entry of LeEix2 into the cell, in an actin- and
238 microtubule-dependent manner. EHD2 has been shown to be linked to the actin
239 cytoskeleton in mammalian cells (Braun et al., 2004; Guilherme et al., 2004), and
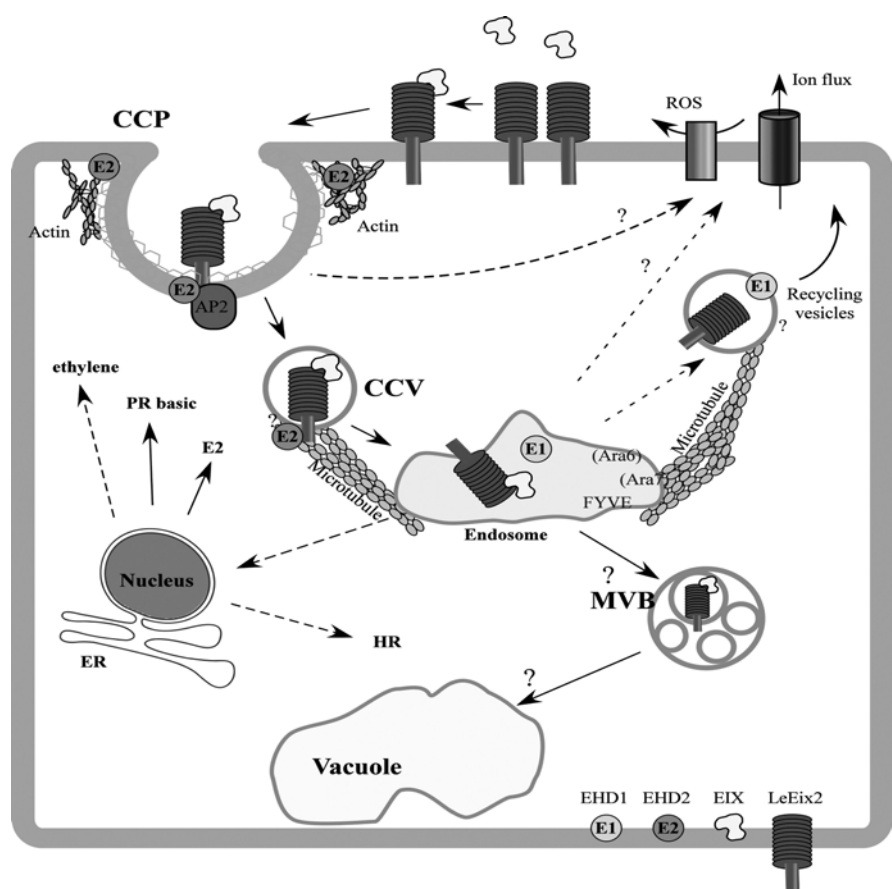
Figure 1. Schematic representation of LeEix localization and putative signaling pathway. Localization of known markers indicated. E1 = AtEHD1, E2 = AtEHD2, CCP = clathrin-coated pit; CCV = clathrin-coated vesicle.

[AU4] we have preliminary evidence that this is the case in plants as well (M. Bar et al., 240
unpublished). 241

LeEix2 is internalized on FYVE-positive endosomes, which may also 242
contain EHD1 (Bar et al., 2008); LeEix2 may be recycled back to the plasma 243
membrane on recycling vesicles (which can also contain EHD1 [Rapaport et al., 244
2006]), as internalization experiments of LeEix2 in the presence of cycloheximide 245
were not significantly different than those conducted without cycloheximide, 246
though LeEix2 did remain on FYVE endosomes for longer periods of time in the 247
presence of cycloheximide. LeEix2 is probably recycled to the plasma membrane 248
via vesicles, a process, which does not obligatorily require protein synthesis but 249
may be amplified by the synthesis of certain proteins involved. LeEix2 may also 250
be degraded via the multivesicular bodies/vacuole pathway, at least in part. 251

252   The internalization of LeEix2 is required for induction of defense responses,
253 including ion flux, ROS production, ethylene, and PR protein synthesis (Bailey
254 et al., 1990, 1992; Laxalt et al., 2007). EIX application also triggers NtEHD2
255 expression, upon which NtEHD2 acts to inhibit the defense response in the short
256 term. Longer exposure to MAMPs leads to a "full-blown" defense response
257 including HR, free of the inhibitory influence of EHD2, suggesting that a control
258 mechanism based on the interplay of different proteins may be at work.

5. Conclusions

260 Plants are continuously exposed to pathogenic microorganisms in their environ-
261 ment, and possess many mechanisms aimed at mounting an effective defense
262 against these pathogens. In many cases, the host plant recognizes foreign molecules
263 associated with the microorganism, termed microbe-associated molecular patterns
264 (MAMPs). Elicitors (MAMPs) that trigger plant defense responses have been iso-
265 lated from a variety of phytopathogenic and nonpathogenic microorganisms (Ebel
266 and Cosio, 1994; Felix et al., 1999; Fuchs et al., 1989; Ricci et al., 1993).
267   Leucine-rich-repeat receptor-like-proteins (LRR-RLP) have been reported
268 to be involved in plants' ability to sense and respond to several microbial patho-
269 gens. The transmembranal receptor-like proteins studied include receptor-like
270 kinases (RLKs) such as FLS2 and EFR and receptor-like proteins (RLPs, lacking
271 a kinase domain) such as the LeEix proteins and the Cf proteins. In the cases of
272 plant defense against fungal pathogens, the plant resistance receptors identified
273 thus far are predominantly receptor-like proteins.
274   LeEix2 mediates the recognition and response to ethylene-inducing xylanase
275 (EIX) elicitor. LeEix2 contains the endocytosis motif Yxxφ. Mutating the Yxxφ
276 motif in LeEix2 abolishes EIX-mediated hypersensitive response, suggesting that
277 endocytosis plays a key role in the signal transduction pathway. Endocytosis has
278 also been demonstrated to be involved in the signaling of additional "anti-fungal"
279 RLPs such as Ve1. We have previously shown that EIX triggers internalization of
280 the LeEix receptor and that plant EHD2 is an important factor in the internaliza-
281 tion and downstream signaling of EIX/LeEix and RLPs of the Cf family.

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Author Queries

Queries	Details Required	Author's Response
AU1	Jones et al., 1994b is not listed. Please check if 1994b can be changed to 1994.	
AU2	Please provide year of unpublished results.	
AU3	Please provide year of unpublished data.	
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