

Article Addendum

EHD2 inhibits signaling of leucine rich repeat receptor-like proteins

Maya Bar and Adi Avni*

Department of Plant Sciences; Tel-Aviv University; Tel-Aviv, Israel

Key words: LRR-RLP, LeEix, Cf β , FLS2, EH domain, endocytosis, hypersensitive response

Leucine-rich-repeat receptor protein (LRR-RLPs) and LRR-RLKs have been linked with signaling and defense responses in plants. EIX (ethylene-inducing xylanase) triggers a defense response via the LeEix2 receptor, while bacterial flagellin triggers plant innate immunity via the FLS2 receptor. Endocytosis has been suggested to be crucial for the process in both cases. Recently, we showed that the EIX elicitor triggers internalization of the LeEix2 receptor. Additionally, we demonstrate that plant EHD2 is an important factor in the internalization and regulation of the induction of plant immunity in the case of EIX/LeEix2 but does not appear to be involved in the flg/FLS2 system. Here we show that EHD2 is also involved in the signaling of the Cf4 and Cf β receptors, causing inhibition of hypersensitive response (HR) and ethylene biosynthesis upon overexpression of EHD2. Our results suggest that different endocytosis pathways are involved in the induction of plant defense responses.

Leucine-rich-repeat receptor kinase (LRR-RLKs) and Leucine-rich-repeat receptor like proteins (LRR-RLPs) have been linked with signaling and defense responses in plants.¹ The most extensively studied LRR-RLK in the context of plant defense responses is FLS2, which recognizes bacterial flagellin and the flagellin-derived peptide flg22.^{2,3} 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.^{2,4,5} Though less information is available concerning LRR-RLPs, they 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.^{6,7} Genetic compatibility of a Cf protein and its Avr counterpart typically leads to defense responses including

oxidative burst, ion fluxes, MAP kinase activation and induction of hypersensitive response (HR)⁸ that inhibits *C. fulvum* proliferation. Additional LRR-RLPs include the tomato Ve resistant proteins^{9,10} and the LeEix proteins.¹¹

Endocytosis is currently considered to be involved in plant immunity in several cases,¹¹⁻¹³ and Microbial elicitors, such as *Verticillium*¹⁴ and EIX¹⁵ have been shown to enter plant cells. Microbial elicitors have also been shown to stimulate endocytosis.^{12,16,17}

We have recently reported that plant EHD2, an EH domain containing protein,¹⁸ is an important factor in the internalization and downstream signaling of EIX/LeEix2 but does not appear to be involved in FLS2 signaling.¹²

Given that AtEHD2 inhibited LeEix2 signaling but did not affect FLS2 or Pto signaling,¹² we wished to examine whether AtEHD2 has a similar effect with the ligands of other pattern recognition receptors which more closely resemble LeEix2 in structure. Cf β and Cf4 were chosen as receptors which most resemble LeEix2 as they are transmembrane receptors with an extracellular LRR and a short cytoplasmic domain containing a classical Yxx ϕ endocytosis motif.^{19,20} Similar to the phenomenon we observed with EIX, HR induction by Avr9 and Avr4 was inhibited by overexpression of AtEHD2 (Fig. 1A). Figure 1B shows that ethylene biosynthesis induced by Avr9 is also inhibited by AtEHD2 overexpression.

In a recent study, we have shown that the endocytic protein EHD2, which we have previously shown to inhibit endocytosis upon overexpression,¹² appears to be an essential component in the endocytosis and signaling of LeEix2, while it does not seem to be involved in the FLS2 system. Here we show that AtEHD2 is also involved in the signaling of the Cf4 and Cf β receptors, causing inhibition of HR and ethylene biosynthesis upon overexpression.

The LRR-RLPs LeEix2, Cf β and Cf4 are involved in the recognition of fungal effectors and possess structural similarities, such as extra-cellular LRR repeats and a short cytoplasmic domain containing a Yxx ϕ endocytosis motif.^{8,11,19} FLS2 which is a receptor-like-kinase (LRR-RLK) has an intra-cellular kinase domain and a non-classical PEST-like endocytosis motif.^{3,17} It is therefore tempting to speculate that EHD2 is a regulatory protein involved in the endocytosis and signaling of LRR-RLPs having a YXX ϕ motif and lacking a kinase domain. EHD2 binds LeEix2,¹² and may affect endocytosis directly, but may also

*Correspondence to: Adi Avni; Department of Plant Sciences; Tel-Aviv University; Tel-Aviv 69978 Israel; Tel.: 972.3.6409840; Fax: 972.3.6410195; Email: lpavni@post.tau.ac.il

Submitted: 05/19/09; Accepted: 05/20/09

Previously published online as a *Plant Signaling & Behavior* E-publication: <http://www.landesbioscience.com/journals/psb/article/xxxx>

Addendum to: Bar M, Avni A. EHD2 inhibits ligand-induced endocytosis and signaling of the leucine rich repeat receptor-like protein LeEix2. *Plant J* 2009; In press; PMID: 19392695; DOI: 10.1111/j.1365-313X.2009.03897.x.

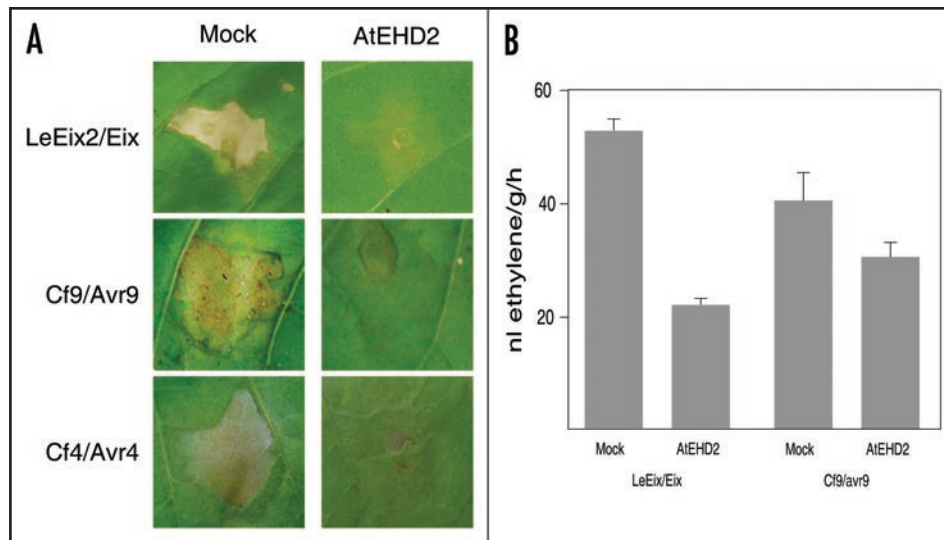


Figure 1. EHD2 overexpression inhibits HR induction and ethylene biosynthesis. (A) *N. tabacum* transiently transformed with Pro_{35S}:tvEIX or a mixture of Pro_{35S}:Cf9 and Pro_{35S}:Avr9 or Pro_{35S}:Cf4 and Pro_{35S}:Avr4 and either Pro_{35S}:AtEHD2 or Pro_{35S}:GFP. Induction of HR was monitored 96 h after transformation. (B) Leaf disks of transiently transformed tobacco leaves with either mock (Pro_{35S}:GFP) or Pro_{35S}:AtEHD2 and Pro_{35S}:Cf9/Pro_{35S}:Avr9 (48 h after transformation), were floated on a 250 mM Sorbitol solution; Leaf disks of transiently transformed tobacco leaves with either mock (Pro_{35S}:GFP) or Pro_{35S}:AtEHD2 were also floated on a 250 mM Sorbitol solution supplemented with 2.5 µg/ml EIX. Ethylene biosynthesis was measured after 4 hours. Error bars represent the average ± SE of 4 different experiments.

modulate internalization of LeEix2 and possibly additional LRR-RLPs through an effect on the plasma membrane. *C. fulvum* AVR proteins, or their Cf receptors, have never been shown to undergo endocytosis. It was also demonstrated in several cases that Cf9 does not directly bind Avr9.²¹ Despite these facts, it would seem that Cf receptors may undergo endocytosis, given that they possess a YXXΦ domain and are capable of having their activity modulated by the endocytic protein EHD2.

FLS2, and possibly additional LRR-RLKs, represent the first line of plant defense which recognizes many pathogens,²² and may be regarded as “broad spectrum”, while *LeEix2*, Cf receptors, and possibly additional LRR-RLPs^{11,19} represent the second line of defense which recognizes specific effectors and induces a faster and stronger response that often culminates in HR induction.²³ Given our results, we suggest that the different specificities in signaling may stem from different interacting proteins which mediate receptor signaling, possibly via different endocytosis pathways. Therefore, EHD2 may grant specificity to different receptor-mediated defense signaling pathways.

A schematic proposed model incorporating this work as well as previous works^{11,15,18,24} is presented in Figure 2. FLS2 is given as an exemplary LRR-RLK, while *LeEix2* is given as an exemplary LRR-RLP. The data relating to FLS2 has previously been published.²² As for *LeEix2*, upon EIX application, EIX binds the *LeEix2* receptor on the outside of the plasma membrane.¹¹

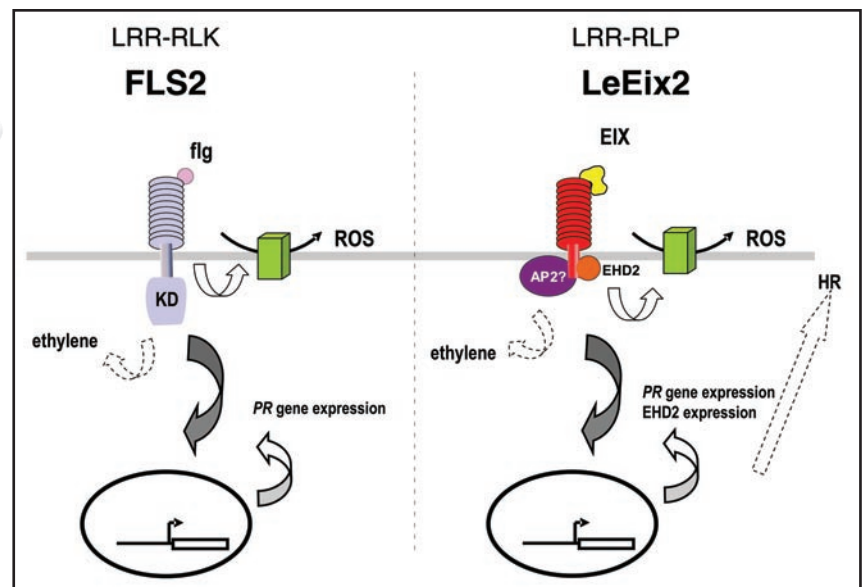


Figure 2. Schematic representation of defense responses mediated by FLS2 (LRR-RLK; left) and *LeEix2* (LRR-RLP; right). FLS2 mediates e.g., ROS production, Ethylene biosynthesis and PR protein synthesis via its kinase domain in response to flagellin (flg₂₂). *LeEix2* interacts with EHD2 and possibly AP-2 and mediates e.g., ROS production, Ethylene biosynthesis, PR protein synthesis, EHD2 expression and HR in response to EIX.

The ligand-receptor complex probably signals for the binding of an endocytic protein complex to the YxxΦ motif present within the cytoplasmic tail of *LeEix2*. One protein in such a complex could be AP-2, which has been shown to bind the YxxΦ motif of transferrin receptor and participate in transferrin internalization in *Arabidopsis* protoplasts.²⁵ Binding of AP-2, usually via the YxxΦ

motif, has also been shown to be a crucial step in the internalization of several mammalian receptors.²⁶ Interestingly, EHD proteins in mammals were shown to bind adaptor proteins, as well as additional proteins of the Clathrin coated vesicle complex.²⁷ This may also be the case in plant cells. It is possible that AP-2 resides in a complex with the cytoplasmic tail of LeEix2 and EHD2, as well as additional proteins. Binding of EIX allows for entry of LeEix2 into the cell, in an actin and microtubule dependent manner. EHD2 has been shown to be linked to the actin cytoskeleton in mammalian cells.^{28,29} The internalization of LeEix2 is required for induction of defense responses, including Ion flux, ROS production, ethylene and PR protein synthesis.³⁰ EIX application also triggers EHD2 expression, upon which EHD2 acts to inhibit the defense response in the short term. Longer exposure to MAMPs leads to a “full-blown” defense response including HR, free of the inhibitory influence of EHD2.

We suggest that at least two different endocytic mechanisms for Pattern recognition receptors exist in plant cells, EHD2 being involved in one such mechanism. EHD2 inhibits signaling of LeEix2, Cf4 and Cf9, LRR-RLP (proteins without a kinase domain) probably by inhibiting their endocytosis and/or inhibiting the internalization of EIX, Avr4 and Avr9 respectively. The endocytic mechanism of FLS2 (LRR-RLK) appears not to involve EHD2.

Acknowledgements

This work was partly supported by German-Israeli BioDisc program and by the Israel Science Foundation administrated by the Israel Academy of Science and Humanities No. 294/07 and Research Grant Award No. 3922-06R from BARD, The United States-Israel Binational Agriculture Research and Development Fund. FYVE-dsRed was a kind gift from Jozef Samaj. We thank Matthieu Joosten and Pierre De Wit for Cf9, Avr9, Cf4 and Avr4 constructs and Gregory Martin and Guido Sessa for Pto and AvrPto constructs. We thank Silke Robatzek and Cyril Zipfel for providing flg22. The authors also wish to thank Mrs. Silvia Schuster and Mrs. Ma'ayan Schwarzkopf.

References

- Torii KU. Leucine-rich repeat receptor kinases in plants: structure, function and signal transduction pathways. *Int Rev Cytol* 2004; 234:1-46.
- Felix G, Duran JD, Volko S, Boller T. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant Journal* 1999; 18:265-76.
- Gomez-Gomez L, Boller T. FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Mol Cell* 2000; 5:1003-11.
- Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, et al. MAP kinase signaling cascade in Arabidopsis innate immunity. *Nature* 2002; 415:977-83.
- Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G, et al. Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* 2004; 428:764-7.
- Takken FLW, Thomas CM, Joosten MHAJ, Golstein C, Westerink N, Hille J, et al. A second gene at the tomato Cf-4 locus confers resistance to *Cladosporium fulvum* through recognition of a novel avirulence determinant. *Plant J* 1999; 20:279-88.
- van der Hooen RAL, Wulff BBH, Rivas S, Durrant MC, van der Ploeg A, de Wit PJGM, et al. Structure-function analysis of Cf-9, a receptor-like protein with extracytoplasmic leucine-rich repeats. *Plant Cell* 2005; 17:1000-15.
- Kruijt M, De Kock MJD, De Wit PJGM. Receptor-like proteins involved in plant disease resistance—Review. *Mol Plant Pathol* 2005; 6:85-97.
- Fradin EF, Zhang Z, Ayala JCJ, Castroverde CDM, Nazar RN, Robb J, et al. Genetic dissection of Verticillium wilt resistance mediated by tomato Ve1. *Plant Physiol* 2009; 150:320-32.
- Kawchuk LM, Hachey J, Lynch DR, Kulcsar F, van Rooijen G, Waterer DR, et al. Tomato Ve disease resistance genes encode cell surface-like receptors. *Proc Natl Acad Sci USA* 2001; 98:6511-5.
- Ron M, Avni A. The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant Cell* 2004; 16:1604-15.
- Bar M, Avni A. EHD2 inhibits ligand-induced endocytosis and signaling of the Leucine Rich Repeat Receptor-like Protein LeEix2. *Plant J* 2009; doi: 10.1111/j.1365-313X.2009.03897.x.
- Robatzek S. Vesicle trafficking in plant immune responses. *Cell Microbiol* 2007; 9:1-8.
- Horn MA, Heinstein PF, Low PS. Receptor-mediated endocytosis in plant cells. *Plant Cell* 1989; 1:1003-9.
- Hanania U, Furman-Matarasso N, Ron M, Avni A. Isolation of a novel SUMO protein from tomato that suppresses EIX-induced cell death. *Plant J* 1999; 19:533-41.
- Leborgne-Castel N, Lherminier J, Der C, Fromentin J, Houot V, Simon-Plas F. The plant defense elicitor cryptogein stimulates clathrin-mediated endocytosis correlated with reactive oxygen species production in Bright Yellow-2 tobacco cells. *Plant Physiol* 2008; 146:1255-66.
- Robatzek S, Chinchilla D, Boller T. Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. *Genes & Dev* 2006; 20:537-42.
- Bar M, Aharon M, Benjamin S, Rotblat B, Horowitz M, Avni A. AtEHDs, novel Arabidopsis EH-domain-containing proteins involved in endocytosis. *Plant J* 2008; 55:1025-38.
- Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ, Jones JD. Isolation of the tomato Cf-9 gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* 1994; 266:789-93.
- Takken FLW, Schipper D, Nijkamp HJJ, Hille J. Identification and Ds-tagged isolation of a new gene at the Cf-4 locus of tomato involved in disease resistance to *Cladosporium fulvum* race 5. *Plant J* 1998; 14:401-11.
- Luderer R, Rivas S, Nurnberger T, Mattei B, Van den Hooven HW, Van der Hooen RA, et al. No evidence for binding between resistance gene product Cf-9 of tomato and avirulence gene product AVR9 of *Cladosporium fulvum*. *Mol Plant Microbe Interact* 2001; 14:867-76.
- Boller T, Felix G. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol* 2009; 60:379-406.
- Jones JD, Dangl JL. The plant immune system. *Nature* 2006; 444:323-9.
- Rotblat B, Enshell-Seiffers D, Gershoni JM, Schuster S, Avni A. Identification of an essential component of the elicitation active site of the EIX protein elicitor. *Plant J* 2002; 32:1049-55.
- Ortiz-Zapater E, Soriano-Ortega E, Marcote MJ, Ortiz-Masia D, Aniento F. Trafficking of the human transferrin receptor in plant cells: effects of tyrphostin A23 and brefeldin A. *Plant J* 2006; 48:757-70.
- Traub LM. Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. *J Cell Biol* 2003; 163:203-8.
- Rotem-Yehudar R, Galperin E, Horowitz M. Association of insulin like growth factor 1 receptor with EHD1 and SNAP29. *J Biol Chem* 2001; 276:33054-60.
- Braun A, Agelet RP, Dahlhaus R, Grant BD, Kessels MM, Qualmann B. Interaction of EHD proteins with syndapin I and II, a basis for functional connections between membrane trafficking and the actin cytoskeleton. *Eur J Cell Biol* 2004; 83:81.
- Guilherme A, Soriano NA, Bose S, Holik J, Bose A, Pomerleau DP, et al. EHD2 and the novel EH domain binding protein EHP1 couple endocytosis to the actin cytoskeleton. *J Biol Chem* 2004; 279:10593-605.
- Laxalt AM, Raho N, ten Have A, Lamattina L. Nitric Oxide is critical for inducing phosphatidic acid accumulation in xylanase-elicited tomato cells. *J Biol Chem* 2007; 282:21160-8.