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The function of EHD2 in endocytosis and defense signaling is affected by SUMO

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Abstract Post-translational modification of target proteins by the small ubiquitin-like modifier protein (SUMO) regulates many cellular processes. SUMOylation has been shown to regulate cellular localization and function of a variety of proteins, in some cases affecting nuclear import or export. We have previously characterized two EHDs (EH domain containing proteins) in Arabidospis and showed their involvement in plant endocytosis. AtEHD2 has an inhibitory effect on endocytosis of transferrin, FM-4-64, and the leucine rich repeat receptor like protein LeEix2, an effect that requires and intact coiled-coil domain. Inhibition of endocytosis of LeEix2 by EHD2 is effective in inhibiting defense responses mediated by the LeEix2 receptor in response to its ligand EIX. In the present work we demonstrate that SUMOylation of EHD2 appears to be required for EHD2-induced inhibition of LeEix2 endocytosis. Indeed, we found that a mutant form of EHD2, possessing a defective SUMOylation site, has an increased nuclear abundance, can no longer be SUMOylated and is no longer effective in inhibiting LeEix2 endocytosis or defense signaling in response to EIX.

Keywords EHD2 · EH domain · Endocytosis · SUMO · SUMOylation · LeEix · EIX

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Introduction

Modification by small ubiquitin-like modifier (SUMO) conjugation has diverse cellular functions. SUMOylation is associated with cell cycle activity, DNA repair, nuclear processes, sub nuclear localization, enzymatic activity and stability, as well as regulation of gene expression (Anckar et al. 2006; Hay 2005; Hilgarth et al. 2004; Miura and Hasegawa 2010; Verger et al. 2003). SUMOylation is required for eukaryote viability in many species including yeast, nematodes, vertebrates, and plants (Fraser et al. 2000; Johnson and Blobel 1997; Nacerddine et al. 2005; Saracco et al. 2007). Typically, only a small fraction of a given substrate is in the SUMOylated form at a given time point, suggesting that SUMO conjugation provides additional/different functionality required for non-standard tasks (Hilgarth et al. 2004; Johnson 2004). SUMO proteins are ubiquitously expressed in eukaryotes. *Saccharomyces cerevisiae, S. pombe* and Drosophila have a single SUMO gene, Smt3, Pmt3 and dSmt3 respectively (Johnson and Blobel 1997; Lehembre et al. 2000; Tanaka et al. 1999), mammalians have 4 SUMO genes SUMO1-4 (Guo et al. 2004; Melchior 2000), and plants have several SUMO genes (Colby et al. 2006; Hanania et al. 1999; Kurepa et al. 2003). Conjugation of SUMO to the target proteins occurs through a series of biochemical steps (Hochstrasser 2009; Kerscher et al. 2006).

Many different mammalian SUMO target proteins have been identified, and SUMOylation has emerged as an important regulatory mechanism for protein function and localization in yeast, animals, and humans (Golebiowski et al. 2009; Kerscher et al. 2006). Several proteins were shown to undergo SUMOylation in Arabidopsis (Budhiraja et al. 2009; Jin et al. 2008; Miura and

Hasegawa 2010;Miura et al. 2005, 2007, 2009). In plants, SUMOylation has a role in stress responses (Kurepa et al. 2003; Mazur and van den Burg 2012; Miura et al. 2007; Park and Yun 2013; Xu and Yang 2013; Yoo et al. 2006), pathogen defense (Hanania et al. 1999; Hotson et al. 2003; Hotson and Mudgett 2004; Matarasso et al. 2005; Roden et al. 2004; Xu and Yang 2013), hormonal signaling (Lois et al. 2003; Park and Yun 2013), flowering induction (Jin et al. 2008; Murtas et al. 2003; Park and Yun 2013; Reeves et al. 2002), regulation of gene expression (Miura and Hasegawa 2010; Miura et al. 2009; Park and Yun 2013) and cellular division and expansion processes (Park and Yun 2013; Xu and Yang 2013). Recent works have shown additional links between SUMOylation and plant defense responses (Elrouby and Coupland 2010; Mazur and van den Burg 2012; van den Burg et al. 2010; Xu and Yang 2013), as well as an involvement for SUMO in nuclear transport (Park and Yun 2013).

Plants require endocytosis for important processes including development (Kleine-Vehn and Friml 2008) and defense against microorganisms (Bar and Avni 2009a, b; Robatzek 2007). The endocytic process involves a large number of protein–protein interactions, including proteins which contain one or more EH domains (Carbone et al. 1997; Wong et al. 1995). The EH domain structure consists of two EF-hands and a helix-loop-helix structure that binds calcium (or a pseudo EF-hand), connected by an anti-parallel beta-sheet (de Beer et al. 2000; Di Fiore et al. 1997; Santolini et al. 1999). EHD Orthologs are found in mammalians, vertebrates and insects. (Blume et al. 2007; George et al. 2007; Guilherme et al. 2004). Overexpression of mammalian EHD2 led to inhibition of transferrin endocytosis. (Guilherme et al. 2004). Mammalian EHD2 and mammalian SUMO were recently shown to interact (Pekar et al. 2012); SUMOylation of mammalian EHD2 affects its localization.

We previously reported the isolation and characterization of two Arabidopsis EH domain containing proteins (AtEHD1 and AtEHD2; Bar et al. 2008). Both proteins contain an EH domain with two EF calcium binding hands, a bipartite NLS and a coiled-coil or helical domain, as well as a Dynamin-N motif (Dynamin like GTPase domain). The two proteins were found to be involved in endocytosis in plant systems, and to possess functions similar to those of mammalian EHDs. AtEHD2 was found to have an inhibitory effect on endocytosis of both FM-4-64 in plant cells and transferrin in mammalian cells (Bar et al. 2008). We have also demonstrated that plant EHD2 binds the cytoplasmic domain of the LeEix2 receptor and inhibits its internalization and signaling (Bar and Avni 2009a, b).

Nuclear functions for mammalian endocytic proteins have recently emerged (Pilecka et al. 2007). One of the mammalian EH-containing proteins, epidermal growth factor receptor substrate 15 (Eps15), and its interactor, Epsin1, were shown to accumulate in the nucleus upon nuclear export inhibition (Pekar et al. 2012; Pilecka et al. 2007). Other endocytic machinery proteins were also documented as nucleocytoplasmic shuttling proteins (Pilecka et al. 2007). Several mammalian endocytic proteins were suggested to have a role in transcription regulation and chromatin remodeling (Pilecka et al. 2007), and mammalian EHD2 was reported to shuttle from the cytoplasm to the nucleus (Pekar et al. 2012).

The fungal protein ethylene-inducing xylanase (EIX) (Dean et al. 1989) induces ethylene biosynthesis, electrolyte leakage, expression of PR proteins and HR in specific plant species/varieties (Bailey et al. 1992, 1990; Elbaz et al. 2002; Ron and Avni 2004). EIX was shown to specifically bind to the plasma membrane of both tomato and tobacco responsive 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 locus, termed LeEix (Ron and Avni 2004). LeEix2 contains the conserved endocytosis signal Yxxϕ within the short cytoplasmic domain, and mutation in this endocytosis motif resulted in abolishment of HR induction in response to EIX (Ron and Avni 2004). We also found EIX to induce endocytosis of LeEix2 (Bar and Avni 2009a, b). As described above, plant EHD2 binds the cytoplasmic domain of the LeEix2 receptor and inhibits its internalization and signaling (Bar and Avni 2009a, b).

In this work we examined the involvement of SUMOylation in EHD2 function in endocytosis and defense responses. We show here that the ability of plant EHD2 to inhibit internalization and signaling of the LeEix2 receptor requires SUMOylation of Lysine480 in EHD2. Mutation of this lysine residue prevents SUMO conjugation to EHD2, results in an increased EHD2 nuclear presence and abolishes the ability of EHD2 to inhibit LeEix2 internalization and signaling.

Materials and methods

Plant material and growth conditions

Nicotiana tabacum cv *samsun* and *Nicotiana benthamiana* were grown from seeds under greenhouse conditions.

Construction of expression vectors

AtEHD2 was cloned in the sense orientation upstream of the *GFP* gene into the binary vector pBINPLUS (van

Engelen et al. 1995) between the 35S-Ω promoter containing the translation enhancer signal and the Nos terminator, generating *Pro*$_{35S}$*:AtEHD2-GFP*. The original cloning vector pBINPLUS containing the GFP gene served as a control where applicable. Primers used to clone AtEHD2 are disclosed in (Bar et al. 2008). The point-mutations of AtEHD2, resulting in K480Q, were generated using site directed mutagenesis with the following primers: FOR: gcatacataatgagtcacctgcagaaggaaatgccagc; REV: gctggcatttccttctgcaggtgactcattatgtatgc. All constructs were cloned in pBINPLUS as described for AtEHD2. The constructs were electroporated into *Agrobacterium tumefaciens* GV3101 and the bacteria used for transient expression assays. Except where indicated otherwise, constructs used herein were cloned into pBINPLUS under the 35S promoter.

Transient transformation

Transient expression was performed as previously described (Ron and Avni 2004). Briefly, the constructs were cloned in pBINplus (van Engelen et al. 1995) and introduced by electroporation into *A. tumefaciens* strain GV3101. *Agrobacteria* were grown in LB medium overnight, diluted into an 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 6 h until OD_{600} reached 0.4–0.5. The *Agrobacterium* culture was diluted to $OD_{600} = 0.05$–0.2, and the suspensions were injected with a needleless syringe into the leaves of 7–8 week old tobacco plants. Leaves were observed for protein expression 24–72 h after injection.

Bimolecular fluorescence complementation (BiFc) analysis

LeEix2 cytoplasmic domain (forward primer: 5′ggggcctttaggctg; reverse primer 5′ctggcggccgctcagttccttagctttccc) was sub-cloned in the Spe1 site of pSY751, downstream of the N-terminal fragment of YFP (YN). *AtEHD2* and *AtEHD2*-K480Q were blunt sub-cloned into pSY752 containing the C-terminal fragment of YFP (YC) (Bracha-Drori et al. 2004). The resulting plasmids, pSY751-LeEix2_CD (YN-LeEix2_CD), pSY752-AtEHD2 (YC-AtEHD2), pSY752-AtEHD2-K480Q (YC-AtEHD2-K480Q) and pSY752-SUMO [YC-SUMO; (Cohen-Peer et al. 2010)] were used for transient expression assays in *Nicotiana benthamiana* leaves. After incubation at 24 °C for 48 h, the epidermal cell layers were viewed under a confocal microscope. All images were taken using similar laser and gain settings. Images were manipulated uniformly with Adobe Photoshop and/or Canvas software. For further information see "Confocal microscopy" section below.

Yeast two-hybrid analysis

We performed the split-ubiquitin yeast two hybrid analysis using the DUAL hunter and DUAL membrane system according to the manufacture guidelines (Dual systems Biotech).

To create the in-frame SUMO fusion construct, we cloned the tomato SUMO (Hanania et al. 1999) gene into the pBT3-SUC vector and AtEHD2 (Bar et al. 2008) in pPR3-N. The vectors constructs were confirmed by DNA sequencing.

In planta SUMOylation

Pro$_{35S}$: EHD2-GFP or Pro$_{35S}$, EHD_K480Q-GFP with or without Pro$_{35S}$: SUMO-HA were transiently expressed in *N. benthamiana* plants. 48 h post transformation triton soluble membrane (TSM) extracts (30 μg per lane) were separated on a 8 % SDS-PAGE gel, transferred to nitrocellulose membranes and probed with mouse monoclonal anti-GFP antibodies (Covance, USA).

To verify the expression of SUMO-HA the membrane was stripped and re-probed with rat monoclonal anti-HA antibodies (Roche, Germany).

Nuclear staining quantification

Each nucleus quantified was imaged in—2 μM z-plane slices. The fluorescence of all slices for each nucleus was quantified, and for each nucleus the slice with the highest mean arbitrary fluorescence units score was chosen for statistical analysis. All nuclei present in each field of both samples were quantified (a minimum of 10 cells for each sample). Cytosolic staining of the same images was also quantified as a further control. Quantification was carried out using ImageJ software. All images for quantification were captured using identical conditions.

Confocal microscopy

Cells were analyzed using a Zeiss LSM-510-Meta confocal laser scanning microscope (Zeiss, Oberkochen, Germany) with the following configuration: 30 mW Argon and HeNe lasers, 458, 477, 488, 514 and 568 maximum lines respectively. All images depict single sections, except where indicated otherwise. All images represent similar sized sections and were taken using similar laser and gain settings. Contrast and intensity for each image were manipulated uniformly using Adobe Photoshop, Canvas and/or ImageJ software.

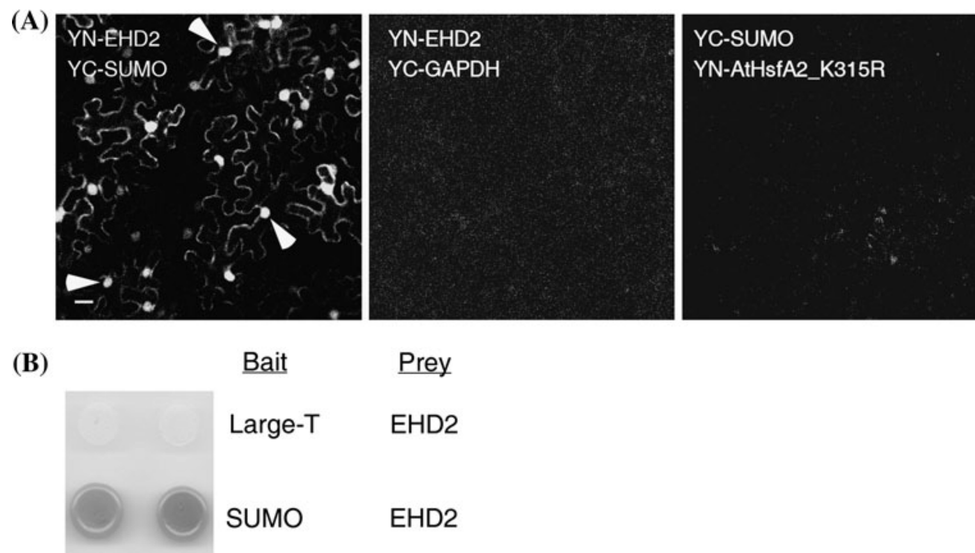

Fig. 1 Interaction between SUMO and AtEHD2. **a** BiFC visualization of the interaction between SUMO and AtEHD2. Fluorescence images of *N. benthamiana* leaves infiltrated with a mixture of *Agrobacterium tumefaciens* (OD_{600} = 0.1) containing Pro_{35S}:YC-SUMO and Pro_{35S}:YN-AtEHD2. Leaf sections were visualized 48 h after injection under a laser-scanning confocal microscope (Zeiss). *Bars* 20 µm. Controls are as indicated in the two *righthand panels*: YN-EHD2 with YC-GAPDH and YC-SUMO with YN-HSFA2. **b** Interactions between AtEHD2 and SUMO yeast cells harboring SUMO (in pBT3-SUC), and AtEHD2 (in pPR3-N) or appropriate controls as indicated, were grown on galactose medium lacking the amino acids tryptophan, leucine, adenine and histidine containing 15 mM 3-AT and supplemented with X-gal

Hypersensitive response and ethylene biosynthesis measurement

Following transient transformation as described above, HR was monitored 48–96 h after transformation, in a minimum of 5 plants.

Ethylene biosynthesis was assayed as described in (Avni et al. 1994). Briefly, leaf discs from transiently transformed *N. tabacum* were incubated for 4 h in 250 mM Sorbitol and 10 mM MES pH 5.7 supplemented with 2.5 µg/ml EIX or un-supplemented. Ethylene was measured after 4 h using a Gas Chromatograph (Varian). Leaves transiently transformed with pBINPLUS expressing GFP under the 35S promoter were used as a control.

Results

Interaction of AtEHD2 with SUMO

We observed EHD2 to be membranal, cytoplasmic and nuclear (Bar et al. 2008; Bar and Avni 2009a, b). Previous work done by us (Matarasso et al. 2005) as well as others (Itahana et al. 2006; Salinas et al. 2004) demonstrates that SUMO can assist in protein shuttling between different cellular locales. Since a high probability ψKxE (in which ψ is an aliphatic branched amino acid and x is any amino acid) SUMOylation site was predicted in AtEHD2 (amino acids 479–482, LKKE), we examined a possible interaction between AtEHD2 and SUMO. A similar interaction was found between mammalian EHD2 and mammalian SUMO (Pekar et al. 2012). Figure 1a demonstrates an interaction between AtEHD2 and SUMO, localized primarily to the nucleus (observe arrowheads) and also to the cytoplasm. The appropriate controls did not result in a fluorescent interaction. This interaction was also confirmed in yeast (Fig. 1b).

In vivo SUMOylation of EHD2

In order to confirm that the predicted SUMOylation site on residues 479–482 is indeed a SUMOylation site, we generated a K480Q mutant of EHD2 and performed in vivo SUMOylation experiments. It has been previously demonstrated that the shift in protein size caused by SUMOylation is detectable (Jin et al. 2008). We therefore proceeded to examine SUMOylation of EHD2 and EHD2-K480Q in protein extracts of plants co-expressing SUMO and the AtEHD2 form as indicated. Figure 2 demonstrates that part of the EHD2 protein can be SUMOylated in an in vivo system, as can be seen by the appropriate-sized shift in the protein migration resulting in a higher band. However, no such shift could be detected in the AtEHD2-K480Q sample. The observed shifted band in Fig. 2 could also be a doublet, since some of the EHD2 in the sample may have remained in its un-conjugated form.

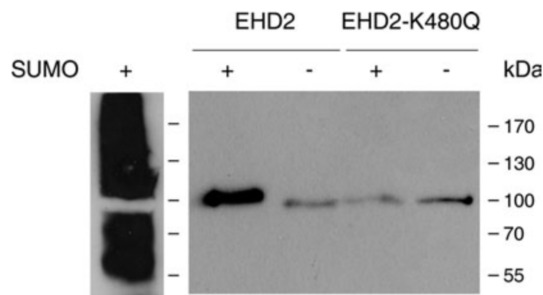

Fig. 2 In planta SUMOylation of AtEHD2/K480Q. Total plant proteins from *N. benthamiana* leaves transiently expressing GFP tagged AtEHD2, GFP tagged AtEHD2-K480Q and SUMO-HA as indicated, were extracted (30 μg/lane) and subjected to 12 % SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-GFP antibodies. Membranes were stripped and re-probed with anti-HA antibodies, a representative *lane* is depicted on the *far left*

Since EHD2 was found to interact with SUMO in the yeast 2-hybrid system (see Fig. 1), we further tested the ability of EHD2-K480Q to interact with SUMO in this system, in order to examine whether K480, which we found to be required for conjugation to SUMO, is also required for the interaction itself. We found that EHD2-K480Q is able to interact with SUMO in the BiFc system and in yeast (supplemental Fig. 1), indicating that lysine 480 is not necessarily required for binding, though it is required for conjugation.

EHD2 mutated in the SUMOylation site has increased nuclear presence

Cellular localization of the SUMO-K480Q mutant indicated that while it is essentially localized similarly to wild-type AtEHD2 (Fig. 3a), it appears to exhibit more strongly stained nuclei. In order to verify this, the GFP staining in nuclei was quantified. Quantification parameters are described in the materials and methods section. As can be seen in Fig. 3b, AtEHD2-K480Q does indeed accumulate in the nucleus as compared with wild-type EHD2—compare arrowheads indicating stained nuclei between the left and right panels of Fig. 3a. Interestingly, the in planta interaction between AtEHD2 and SUMO appears to be predominantly nuclear (Fig. 1), suggesting that at least part of the AtEHD2 SUMOylation occurs in the nucleus. The cytosolic staining of both wild-type and mutant EHD2 was also quantified and found to be of comparable levels.

Endocytosis of LeEix2 in response to EIX treatment in the presence of AtEHD2-K480Q

We have previously shown that AtEHD2 inhibits the internalization of transferrin, FM-4-64 (Bar et al. 2008) and

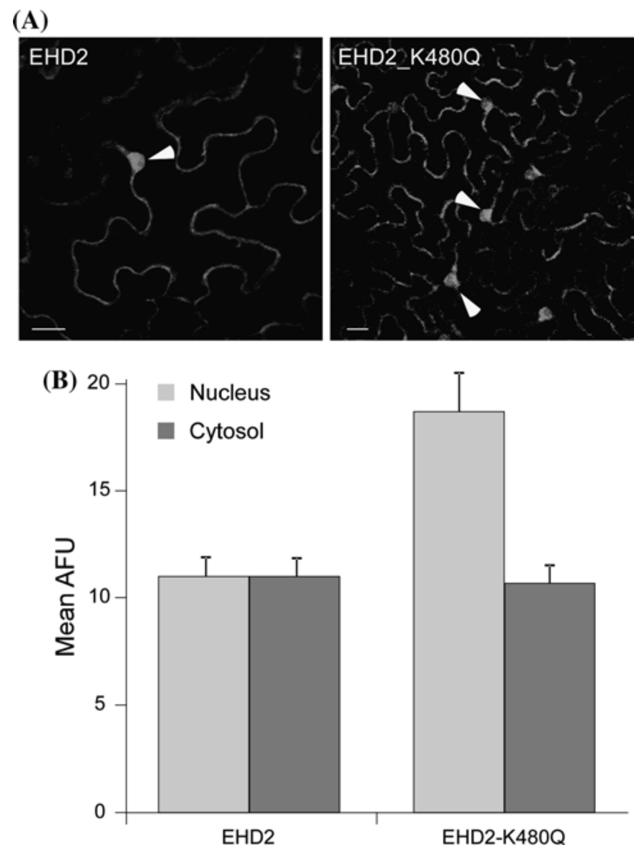

Fig. 3 Localization and nuclear quantification of AtEHD2 and AtEHD2-K480Q. **a** *N. benthamiana* leaves transiently expressing GFP tagged EHD2 or GFP tagged EHD2-K480Q as indicated, were visualized 48 h after transformation under a laser-scanning-meta confocal microscope (zeiss). *Bars* 10 μm. **b** GFP fluorescence from at least 10 cells of each sample was quantified using ImageJ software. GFP was quantified using a captured 2 μM slice; both the nucleus and the cytosolic fluorescence were quantified in all panels (for the nucleus, the slice representing the highest GFP fluorescence within each nucleus respectively was quantified for each cell). All images were captured using identical conditions

LeEix2 (Bar and Avni 2009a, b). We examined whether AtEHD2 mutated in its SUMOylation site retained the ability to inhibit LeEix2 endocytosis in response to EIX treatment. As we have previously described in several instances, LeEix2 is internalized on FYVE-positive endosomes (Bar and Avni 2009a, b). Briefly, we see GFP-tagged LeEix2 on FYVE-positive endosomes 10–15 min after EIX application, while GFP-tagged LeEix2 has low co-localization with the FYVE marker in untreated leaves [~90 % in EIX treated leaves as compared with ~30 % in untreated leaves (Bar and Avni 2009a, b)]. The FYVE domain has been reported to localize to endosomes in mammals (Stenmark et al. 1996) as well as plants (Heras and Drobak 2002; Jensen et al. 2001; Voigt et al. 2005). Figure 4 demonstrates that while over-expression of AtEHD2 inhibits LeEix2 endocytosis in response to EIX treatment (observe red

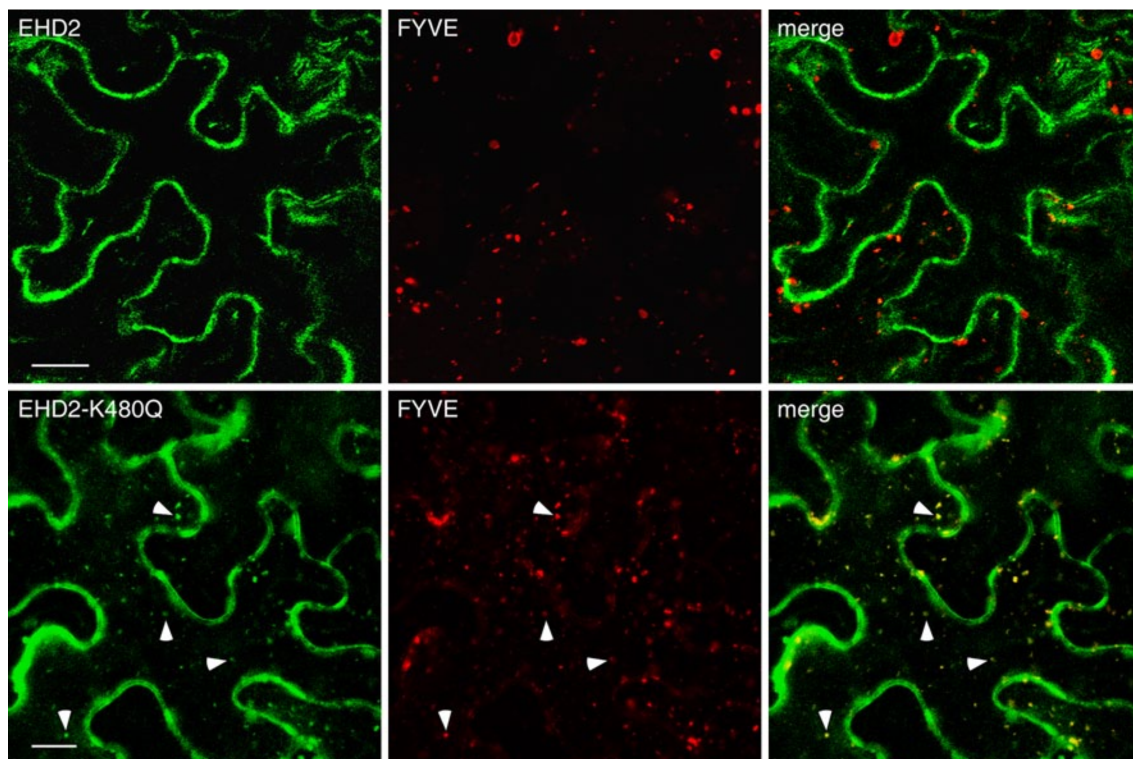

Fig. 4 GFP-LeEix2 internalization 15 min after EIX application in the presence of EHD2 or EHD-K480Q. *N. benthamiana* transiently expressing LeEix2, FYVE-dsRed and AtEHD2-GFP or AtEHD-K480Q-GFP as indicated were treated with EIX (2.5 µg/g tissue) by petiole application, and visualized 15 min after treatment. *Bars* 20 µm

endosomes lacking in LeEix2 presence), EHD2-K480Q has lost this inhibitory activity (observe yellow endosomes on which LeEix2 is present, also indicated by arrowheads). Interestingly, while high expression of mammalian EHD2 leads to inhibition of transferrin internalization (Benjamin et al. 2011; Blume et al. 2007; Guilherme et al. 2004), overexpression of mutant EHD2 which accumulates in the nucleus (EHD2KK315-316AA), is no longer able to block transferrin internalization (Pekar et al. 2012).

EIX signaling in the presence of EHD2-K480Q

We have demonstrated that over-expression of AtEHD2 inhibits EIX induction of ethylene biosynthesis and induced cell death (HR), as well as other downstream defense responses (Bar and Avni 2009a, b). To test for the effect of AtEHD2-K480Q on ethylene biosynthesis, *N. tabacum* leaves were transiently transformed with *Pro*$_{35S}$*: AtEHD2-GFP* or *Pro*$_{35S}$*: AtEHD_K480Q-GFP*; *Pro*$_{35S}$*:GFP* served as a control. Forty-eight hours after transformation, leaf discs were prepared from the injected leaves and incubated with 2.5 µg/ml EIX. Ethylene production was measured after 4 h of incubation. EHD2 greatly reduces the amount of ethylene produced in response to EIX, (Fig. 5a; Bar and Avni 2009a, b), while EHD2-K480Q has lost this inhibitory activity.

Moreover, over-expression of EHD2-K480Q failed to inhibit HR induction by EIX (Fig. 5b), similar to the loss of the ability to inhibit the induction of ethylene biosynthesis.

Interaction of EHD2-K480Q with LeEix2

We have previously demonstrated that AtEHD2 interacts with the cytoplasmic domain of LeEix2 *in planta* in the BiFc system (Bar and Avni 2009a, b). Here we examined reconstitution of YFP fluorescence by transient co-expression of AtEHD2-K480Q and the cytoplasmic domain of LeEix2 (LeEix2_CD) in *N. benthamiana* leaves. In Fig. 6 in the left-hand panel are depicted cells co-expressing YN-LeEix2_CD and YC-AtEHD2_K480Q, which show YFP fluorescence localized to the cell membrane. The proteins were also examined for fluorescence with the complementary half of the YFP protein and the results were negative (Bar and Avni 2009a, b). Our results demonstrate that SUMOylation is not required for EHD2 binding to LeEix2, though it may be involved in LeEix2 internalization. Interestingly, the interaction of wild-type EHD2 with the LeEix2 receptor appears to

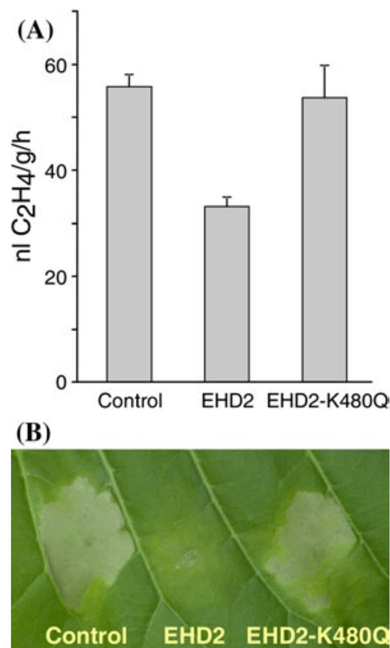

Fig. 5 Effect of over-expression of AtEHD2/K480Q on EIX-induced defense responses. *N. tabacum* transiently transformed with a mixture of tvEIX and AtEHD2 forms as indicated. **a** Leaf disks of control (GFP), AtEHD2 or AtEHD2-K480Q as indicated (48 h after transformation), were floated on a 250 mM Sorbitol solution with 2.5 μg/ml EIX. Ethylene biosynthesis was measured after 4 h. *Error bars* represent the average + SE of 4 different experiments. **b** Induction of HR was monitored 48 h after transformation

be more punctate within the cell membrane, while the interaction of EHD2-K480Q with the LeEix2 receptor appears smoother and more uniform. Thus, while SUMOylation is not strictly required for this interaction, it may affect the precise localization of the interaction or the ability of the complex to reside in specific micro-domains within the cellular membrane. Since EHD2-K480Q is no longer able to inhibit endocytosis, it is possible that localization to these microdomains is functionally relevant for endocytosis of LeEix2.

Discussion

We previously showed that wild-type AtEHD2 is an endocytosis inhibitory protein, inhibiting internalization of a variety of cargo such as transferrin and FM-4-64, as well as ligand-induced endocytosis of—and signaling mediated by—the LeEix2 receptor (Bar et al. 2008; Bar and Avni 2009a, b). Additional research has shown that wild type mammalian EHD2 can inhibit endocytosis (Guilherme et al. 2004).

In this work we demonstrate that SUMOylation of plant EHD2 is most probably required for inhibition of endocytosis of the LeEix2 receptor and downstream signaling mediated by the receptor.

We further demonstrate that SUMOylation is involved in shuttling EHD2 from the nucleus, as EHD2 mutated in its SUMOylation site accumulates in the nucleus to a certain degree. SUMO regulation of nuclear—cytoplasmic shuttling was demonstrated for a plant cysteine protease (Matarasso et al. 2005), as well as for mammalian EHD2 (Pekar et al. 2012).

Therefore, we would like to propose the model presented in Fig. 7: EHD2 is present in the nucleus, where it may act as a transcriptional repressor (Pekar et al. 2012). Upon SUMOylation, EHD2 exits the nucleus and shuttles to the membrane, where it binds to LeEix2 [and probably other receptors the signaling of which is regulated by endocytosis, see for example (Bar and Avni 2009a, b)] and inhibits LeEix2/EIX endocytosis. EHD2-K480Q cannot undergo SUMoylation, does not exit the nucleus (or exits

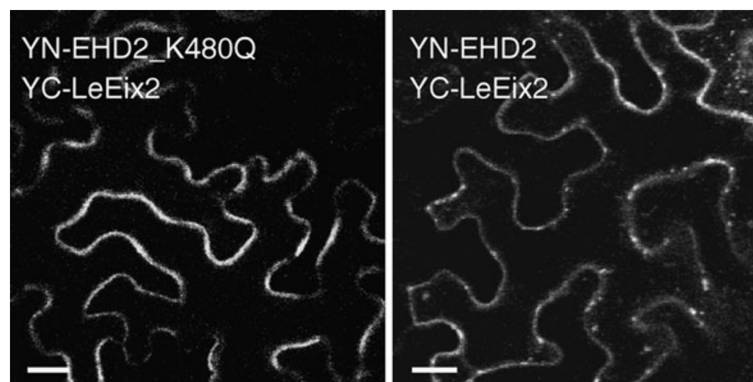

Fig. 6 BiFC visualization of the interaction between AtEHD2 and LeEix2. Fluorescence images of *N. benthamiana* leaves infiltrated with a mixture of *Agrobacterium tumefaciens* (OD_{600} = 0.1) containing Pro_{35S}:YN-LeEix2-CD (Bar and Avni 2009a, b) and Pro_{35S}:YC-AtEHD2 or Pro_{35S}:YC-AtEHD2-K480Q. Leaf sections were visualized 48 h after injection under a laser-scanning confocal microscope (Zeiss). *Bars* 20 μm

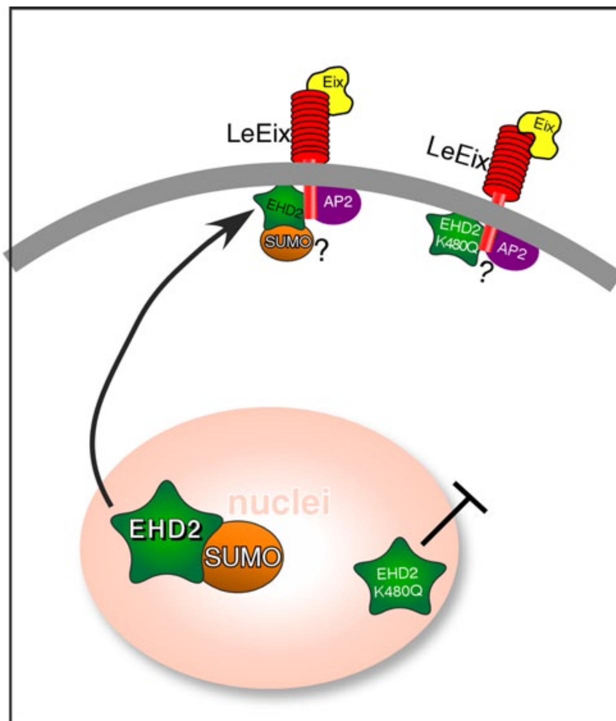

Fig. 7 Schematic model proposing a possible mechanism for the interaction between LeEix2 EHD2 and SUMO. EHD2 resides in the nucleus, and can only exit the nucleus upon SUMOylation. Following SUMOylation of EHD2 in the nucleus, EHD2 (either alone or in complex with SUMO) exits the nucleus and is trafficked to the plasma membrane. In the plasma membrane, upon binding of EIX to the LeEix2 receptor, LeEix2 is tethered to the adaptin complex via binding of the μ-adaptin subunit (AtAP-2 μ; At5g46630) to its YXXϕ motif. EHD2 binds σ-adaptin (AtAP-2 σ; At2g19790) via its coiled-coil domain, subsequently inhibiting internalization of LeEix2 and EIX derived signaling

to a much lower degree) and does not inhibit endocytosis and signaling of LeEix2. In the case of EHD2-K480Q overexpression, EHD2-K480Q also resides in the cytosol/membrane, where it is also able to bind LeEix2.

Our results are also supported by a recent work done in mammalian cells, demonstrating that mammalian EHD2 shuttles from the membrane/cytoplasm to the nucleus, where it probably acts as a transcription co-repressor. This work also demonstrated that the exit of EHD2 from the nucleus depends mainly on its SUMOylation at Lysine 315. Interestingly, SUMOylation was also shown to promote endocytosis of a mammalian receptor (Martin et al. 2007); we also show here that SUMOylation of EHD2 is required for LeEix2 internalization, as it would seem that the level of EHD2-K480Q at the plasma membrane is still high, despite its increased nuclear presence.

SUMO was shown to be involved in defense responses in a few plant systems (Mazur and van den Burg 2012; Park and Yun 2013; Saracco et al. 2007; Xu and Yang 2013). It has also been demonstrated that the level of SUMOylated proteins increases in response to various cellular stresses, including pathogens (Cohen-Peer et al. 2010). We have previously demonstrated that plants overexpressing SUMO have suppressed defense responses following EIX treatment, (Hanania et al. 1999). This could potentially stem from SUMOylation of various proteins involved in defense and or protein/protein interactions, the SUMOylation of which serves to somehow inhibit defense, possibly via inhibition of receptor internalization. This presents an interesting parallel between SUMO and EHD2—overexpression of both (each individually) inhibits EIX induced defense. Though we demonstrate herein that EHD2 undergoes SUMOylation, which is required for its inhibition of EIX induced defense, additional experimentation is required in order to determine whether SUMO-derived inhibition of defense responses stems from SUMOylation of EHD2 (and probably additional proteins like EHD2 involved in LeEix2 endocytosis and/or signaling) or whether there are additional SUMO targets/mechanisms at play which cause inhibition of EIX induced defense as a result of SUMO overexpression.

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