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SlRLK-like is a malectin-like domain protein affecting localization and abundance of LeEIX2 receptor resulting in suppression of EIX-induced immune responses

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Summary

The first line of plant defence occurs when a plant pattern recognition receptor (PRR) recognizes microbe-associated molecular patterns. Plant PRRs are either receptor-like kinases (RLK), which have an extracellular domain for ligand binding, a single-pass transmembrane domain, and an intracellular kinase domain for activating downstream signalling, or receptor-like proteins (RLP), which share the same overall structure but lack an intracellular kinase domain. The tomato LeEIX2 is an RLP that binds ethylene-inducing xylanase (EIX), a fungal elicitor. To identify LeEIX2 receptor interactors, we conducted a yeast two-hybrid screen and found a tomato protein that we termed SlRLK-like. The interaction of LeEIX2 with SlRLK-like was verified using co-immunoprecipitation and a bimolecular fluorescence complementation assay. The defence responses induced by EIX were markedly reduced when SlRLK-like was over-expressed in *Nicotiana benthamiana* or *Nicotiana tabacum*, and mutation of *slrlk-like* using CRISPR/Cas9 increased EIX-induced ethylene production and *SlACS2* (1-aminocyclopropane-1-carboxylate synthase) gene expression in tomato. Co-expression of SlRLK-like with LeEIX2 led to a reduction in its abundance, apparently through an endoplasmic reticulum-associated degradation process. Notably, truncating SlRLK-like protein, revealed that the malectin-like domain is sufficient and essential for its function. Moreover, SlRLK-like associated with RLK FLS2 resulting in its degradation and concomitantly a reduction of flg22-induced burst of reactive oxygen species. In addition, SlRLK-like co-expression with other RLP, Ve1 and AtRLP23, also led to a reduction in their abundance. Our findings suggest that SlRLK-like leads to a decreased stability of various PRRs, leading to their abundance reduction and resulting in attenuation of defence responses.

Introduction

All higher plants possess an innate immune system that is activated after recognition of conserved microbial-derived molecules such as microbe-associated molecular patterns (MAMPs) or pathogen effector proteins (Dangl *et al.* 2013, Thomma *et al.* 2011, Win *et al.* 2012). MAMPs 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). Hallmarks of MAMP-triggered immunity include MAPK kinase and calcium-dependent protein kinase activation, defence gene activation, oxygen radical production, and callose deposition (Boudsocq *et al.* 2010, Thomma et al. 2011). Recognition of pathogen effectors by specific pattern recognition receptors

(PRRs) can also lead to programmed cell death, termed the hypersensitive response (HR) (Thomma et al. 2011).

The key players in MAMP-triggered immunity initiation are PRRs, which act as the plant's first line of defence. Two types of PRRs perceive MAMPs and extracellular effectors: receptor-like proteins (RLPs) and receptor-like kinases (RLKs) (Bohm *et al.* 2014, Zipfel 2014). These receptors often contain extracellular leucine-rich repeats that are thought to monitor ligand presence. Although RLPs and RLKs share an overall similar structure, RLKs contain a cytoplasmic kinase domain, whereas RLPs lacks a kinase domain and have no other obvious signalling domains that would enable transduction of the downstream immune response.

Since RLPs do not possess an obvious signalling domain, the mechanism for initiating downstream cascades has remained elusive. It has been suggested that RLPs form a complex with other proteins to initiate downstream signalling (Bohm et al. 2014, Liebrand *et al.* 2013), as has been proposed for the RLP-CLAVATA complex that mediates maintenance of a meristematic stem cell population (Lee *et al.* 2012, Zhu *et al.* 2010). The RLK SOBIR1 also forms complexes with various RLPs. These RLPs, include Cf proteins, Ve1, LeEIX2 which are involved in immunity and CLAVATA2 (CLV2) and TOO MANY MOUTHS (TMM) which are involved in development (Liebrand et al. 2013). Importantly, SOBIR1 is required for Cf-2.2-, Cf-4-, Ve1- and RLP30- immune responses (Liebrand et al. 2013, Zhang *et al.* 2013). Together this data demonstrate the essential role SOBIR1 epitomises for RLPs activity.

The RLP LeEIX2 mediates the perception of the fungal protein ethylene-inducing xylanase (EIX) in tobacco and tomato (Bailey *et al.* 1993, Ron and Avni 2004). Application of EIX to tobacco or tomato leaves activates various defence responses such as ethylene biosynthesis, reactive oxygen species (ROS) burst, and the HR (Bailey *et al.* 1990, Ron and Avni 2004). Interestingly, the binding of EIX to the LeEIX2 induces receptor-mediated endocytosis and generates the signal needed for the initiation of defence responses (Ron and Avni 2004, Sharfman *et al.* 2011).

We identified a LeEIX2 interactor that shows homology to a member of the CrRLK1L subfamily in *Arabidopsis* (At5g24010) (Boisson-Dernier *et al.* 2011). Members of the CrRLK1L subfamily are found in several plant species such as *Catharanthus roseus* (Schulze-Muth *et al.* 1996),

Platanus × *acerifolia* (Pilotti *et al.* 2014), *Oryza sativa* (Nguyen *et al.* 2015) and tomato (Sakamoto *et al.* 2012). Recent evidence suggests that this subfamily plays an important role in plant defence response activation (Boisson-Dernier et al. 2011, Lindner *et al.* 2012, Xiang *et al.* 2008), in the regulation of cell expansion (Galindo-Trigo *et al.* 2016), and the sensing of cell wall integrity (Engelsdorf and Hamann 2014). Proteins of this subfamily contain a C-terminal cytoplasmic kinase domain and a putative ligand-binding N-terminal region that contains a malectin-like domain (Lindner et al. 2012, Nissen *et al.* 2016). Malectin was first identified as an endoplasmic reticulum (ER) membrane lectin in *Xenopus laevis*, but it is also present in other animals, and it binds carbohydrates with selectivity for diglucose and high mannose N-glycans (Schallus *et al.* 2008). Mammalian malectin participates in ER quality control for glycoproteins (Galli *et al.* 2011, Tannous *et al.* 2015). Although plant CrRLK1L subfamily members contain a malectin-like domain, they are not thought to be involved in N-glycosylation due to the weak conservation of residues in animal malectin that mediate the interaction with glucose (Lindner et al. 2012). Nonetheless, the malectin-like domain is preserved in the CrRLK1L of *Arabidopsis*, which implies an essential role and possibly a conserved ligand-binding capacity (Nissen et al. 2016). Here we report the identification of SlRLK-like which contains a malectin-like domain, as a novel LeEIX2 interactor and show that it acts as a negative regulator of defence responses mediated by the LeEIX2-RLP by significantly reducing its abundance.

Results

Identification of a novel LeEIX2 interactor using yeast two-hybrid methodology

A yeast two-hybrid screen was conducted using *LeEIX2* as bait and an expressed tomato cDNA library as prey. Since the *LeEIX2* gene product is an integral membrane protein, we employed the Split-ubiquitin system (Iyer *et al.* 2005), and we obtained several potential interactors (Supporting Information Table S1). We focused on Solyc01g094920 as bioinformatic analysis and phylogenetic tree assembly revealed that it is an orthologue of a receptor-like protein kinase from *Arabidopsis* (At5g24010), which belongs to the CrRLK1L family (Figure 1a). This protein family was shown to be involved in plant immunity (Boisson-Dernier et al. 2011, Lindner et al. 2012, Xiang et al. 2008). As previously mentioned, LeEIX2-RLP lacks obvious signalling domains that can activate immune signalling. Thus it has been suggested that RLPs form a complex with other

proteins, that posses signalling domains, such as kinases, to initiate downstream signalling (Albert *et al.* 2015, Bohm et al. 2014, Liebrand et al. 2013). CrRLK1L proteins contain both a malectin-like domain and a kinase domain. Solyc01g094920 contains only a malectin-like domain, and therefore we termed it SlRLK-like (Figure 1b). To verify the interaction, direct interaction assays were performed using the original fragment found in the screen (233 amino acids out of the 474 at the C terminus of the protein) or the full-length SlRLK-like protein with LeEIX2 as bait. An empty pPR3-N (prey vector) was used as a negative control. Both the C-terminal fragment and the full-length SlRLK-like gave a positive interaction with LeEIX2, whereas the negative control did not (Figure 1c). Moreover, SlRLK-like did not interact with the associated plasma membrane protein, FLOT1, involved in endocytosis (Li *et al.* 2012), (Supporting Information Figure S1).

SlRLK-like interacts with LeEIX2 and alters its subcellular localization

To confirm that SlRLK-like interacts with LeEIX2 *in planta*, CoIP was performed. We transiently co-expressed SlRLK-like-HA with LeEIX2-GFP or FLOT1-GFP in *N. benthamiana* and pulled down GFP-tagged proteins with anti-GFP beads (Figure 2a). SlRLK-like-HA was successfully pulled down in the presence of LeEIX2-GFP but not when using FLOT1-GFP (a plasma membrane-associated protein) as a control, verifying the assay specificity (Figure 2a). Although LeEIX2-GFP is weakly detected in the input fraction, it is highly concentrated in the IP fraction, indicating an efficient pull-down.

The interaction was further confirmed *in planta* using a bimolecular fluorescence complementation assay (BiFC) (Bracha-Drori *et al.* 2004). *N. benthamiana* epidermal cells co-expressing YN-SlRLK-like and YC-LeEIX2 showed clear YFP fluorescence (Figure 2b). In contrast, no fluorescence was observed when YN-SlRLK-like was co-transformed with YC-FLOT1 or the YC moiety alone (Figure 2b). SlRLK-like co-localized with HDEL, a well-known marker for ER (Nelson *et al.* 2007) (Figure 2c), tagged with mCherry, corroborating the observation of a reticular pattern in the BiFC assay. GFP-tagged LeEIX2 was observed on the plasma membrane and in endosomes (Figure 2d, upper panels) as expected based on previous work (Bar *et al.* 2009, Sharfman et al. 2011) and co-localized with the plasma membrane

associated proteinFLOT1, which served as control for our experiments, when they were co-expressed (Figure 2d). When LeEIX2-GFP was co-expressed with SlRLK-like-mCherry, they co-localized and remarkably, LeEIX2-GFP subcellular localization was modified and apparently retained in ER-like structures (Figure 2d). This was further confirmed when LeEIX2-GFP was co-expressed with HDEL- mCherry and in the absence of SlRLK-like no change in LeEIX2 subcellular localization was observed. Co-expression of LeEIX2 and FLS2-mCherry, did not change LeEIX2 localization (Figure 2d). Moreover, SlRLK-like did not change the localization of FLOT1-mCherry did not co-localize with it, as expected for an ER protein (Figure 2c).

EIX-induced defence responses are reduced in *N. tabacum* transiently over-expressing SlRLK-like

The association of SlRLK-like with LeEIX2 *in planta* suggests an involvement of SlRLK-like in the EIX-LeEIX2 signalling pathway. Ethylene biosynthesis and ROS production are two plant defence responses induced by EIX (Avni *et al.* 1994, Bailey et al. 1990). Over-expression of SlRLK-like reduced the amount of ethylene production by approximately 25% and ROS burst by 56%; both statistically significant differences compared to controls (Figure 3a,b). Moreover, HR development was noticeably delayed in leaves overexpressing SlRLK-like compared to control (Figure 3c). HR was clearly visible in the control at 24 hours post infiltration (hpi), whereas when SlRLK-like-HA was expressed HR was not observed until around 35 hpi. Furthermore, HR progression when SlRLK-like was over-expressed was considerably slower and weaker compared to control (Figure 3c).

The malectin region of SlRLK-like strongly interacts with LeEIX2

Of the 474 amino acids that make up SlRLK-like, 360 amino acids are part of the malectin-like domain. In order to determine the significance of this domain to its interaction with LeEIX2, two versions of the protein were expressed: one consisting of the malectin-like domain (termed malectin-only) and the other consisting of non-malectin tail (Figure 1b). CoIP experiments were performed from extracts of *N. benthamiana* that transiently expressed one of these constructs and LeEIX2. The malectin-only version strongly interacted with LeEIX2, whereas no interaction was observed for the non-malectin tail (Figure 4a). We examined the effect of the truncated versions over-expression on EIX-induced ethylene production and ROS burst production following EIX elicitation. Similar to the full-length SlRLK-like, the malectin-only domain decreased ethylene

production by about 30% and ROS production by about 60% compared to the control (Figure 4b,c). The non-malectin tail had no significant effect on either ethylene or ROS production as compared to the control (Supporting Information Figure S2). Thus, the physiological effect of the truncated versions agrees with their differential interaction to LeEIX2.

Stable tomato lines with null mutations in *slrlk-like* have elevated production of ethylene in response to EIX

Since SlRLK-like over-expression led to reduced EIX-induced defence responses, we generated SlRLK-like mutant plants using CRISPR/Cas9 editing technology and studied their defence response phenotype. Two different edited homozygous lines resulting in truncated SlRLK-like proteins were generated: *slrlk-like3b-3* and *slrlk-like6b-3*, which encode proteins of 142 and 72 amino acids, respectively, instead of the 474 amino acids of the full-length SlRLK-like (Supporting Information Figure S3). The edited lines were examined for ethylene production following application of EIX. Ethylene production increased by about 3.5 fold in the mutant plants compared to transgenic non-edited tomato plants (control) (Figure 5a). *SlACS2* is a key regulatory enzyme that catalyses the formation of an ethylene precursor (Boller *et al.* 1979). It is a member of a divergent multigene family in which each gene is differentially affected by various environmental and developmental factors. *SlACS2* was found to be highly induced by EIX treatment (Matarasso *et al.* 2005). qRT-PCR-based quantification of *SlACS2* expression in edited lines was significantly elevated compared to that in the transgenic non-edited tomato plant (control) following EIX application (Figure 5b) in accordance with the direct measurement of ethylene production.

LeEIX2 abundance decreases when co-expressed with SlRLK-like

As SlRLK-like interacts with LeEIX2 and modifies its subcellular localization, we wanted to investigate the effect of full SlRLK-like and its truncated versions (malectin-only and non-malectin tail) on LeEIX2 expression levels. LeEIX2-GFP was transiently co-expressed with either SlRLK-like-mCherry, malectin-only-mCherry, non-malectin tail-mCherry or free mCherry as control, and protein levels were detected at 48 hours by western blot. LeEIX2 levels were reduced when co-expressed with SlRLK-like-mCherry and malectin-only-mCherry and remained unaffected when co-expressed with non-malectin tail-mCherry as compared to control (Figure 6a). The same trend in LeEIX2-GFP expression was observed in live cells visualized by confocal

microscopy (Figure 6b). Quantification of LeEIX2-GFP expression level by measuring pixel intensity when co-expressed with SlRLK-like and its truncated versions revealed a significant expression decrease when co-expressed with SlRLK-like-mCherry or malectin-only-mCherry (Figure 6c). To determine whether this reduction resulted from differences at the transcriptional or protein level, mRNA was extracted for qRT-PCR analysis. No significant difference in *LeEIX2* mRNA levels was observed between control and cells expressing SlRLK-like (Supporting Information Figure S4), therefore, we presume that LeEIX2 protein abundance is reduced in the presence of SlRLK-like (Figure 6). To determine whether SlRLK-like affects PRRs other than LeEIX2, we examined the effect of SlRLK-like co-expression on levels of two other RLPs, *Verticillum* sp race 1 receptor (Ve1) from *S. lycopersicum* (Kawchuk *et al.* 2001) and RLP23 from *A. thaliana* (Bi *et al.* 2014), and the well-known flagellin receptor RLK FLS2 (Boller *et al.* 2000). SlRLK-like co-expression resulted in lower levels of Ve1 and RLP23 than in cells that expressed the control mCherry (Supporting information Figure S5) FLS2 also decreased compared with the levels in control cells (Supporting information Figure S5). The protein level of the control protein FLOT1 did not change when over expressed with SlRLK-like (Supporting information Figure S5).

Autophagy-related gene 5 (ATG5) silencing results in reduced SlRLK-like-mediated LeEIX2 abundance reduction

In order to further investigate the mechanism by which SlRLK-like leads to the reduction of LeEIX2 protein abundance, we examined whether disrupting autophagy pathways could alter SlRLK-like-mediated degradation. Autophagy begins with the formation of double-membrane vesicle termed autophagosome. The autophagosomes, found in the cytosol, can envelope unwanted cytoplasmatic components and drive them to degradation on the lytic vacuole (Have *et al.* 2019). Autophagy-related gene 5 is one of the core components of the macroautophagy machinery in plants and adjoined with ATG12 (ATG5-ATG12 conjugation system), is required for the formation of ATG8-PE (phosphatidylethanolamine) conjugate, essential for autophagosome biogenesis (Masclaux-Daubresse *et al.* 2017). Mutants of ATG5 gene suffer from reduced autophagy (Fan *et al.* 2019). We generated *N. benthamiana* plants ATG5-silenced using VIGS methodology (Figure 7a), and co-expressed LeEIX2-GFP with either SlRLK-like or FLOT1-mCherry in ATG5-silenced and non-silenced plants. Analysis of confocal images revealed, LeEIX2-GFP expression level, measured by pixel intensity, when co-expressed with

SlRLK-like, was significantly higher in ATG5-silenced plants as compared to non-silenced plants (Figure 7b,d). As opposed, LeEIX2-GFP expression level did not differ significantly when co-expressed with FLOT1-mCherry, between ATG5-silenced and non-silenced plants (Figure 7c,e). Protein levels comparison, detected by western blot, agreed with the analysis of the confocal images, i.e LeEIX2 protein abundance remained almost un-affected when co-expressed with SlRLK-like in ATG5-silenced plants (Figure 7f). It is noteworthy to mention that expression of both SlRLK-like and FLOT1 did not differ between ATG5-silenced and non-silenced plants as can be observed in the immunoblot (Figure 7c) and by comparing their pixel intensity (Supporting information, Figure S6)

SlRLK-like interacts with the RLK FLS2 and attenuates flg22-induced ROS

SlRLK-like co-expression with different RLPs or RLKs results in the reduction of their abundance (Supporting information, Figure S5) we sought out to determine whether SlRLK also interacts with and attenuates the defence responses these proteins initiate. For these experiments, we chose to use the RLK FLS2. The interaction of FLS2 with with SlRLK-like was tested *in planta* using CoIP. SlRLK-like was found to associate with FLS2 (Figure 8a), and, in addition, its presence reduced flg22-induced ROS production by up to 50% relative to samples that expressed the control mCherry (Figure 8b). Similarly, SlRLK-like was also found to associate *in planta* with two additional RLPs: Ve1 and RLP23 (Supporting Information Figure S7). Thus, our data suggest that SlRLK-like could participate in the down regulation of defence responses initiated both by RLPs (LeEIX2) and RLKs (FLS2) by associating with PRRs and leading to a reduction of their abundance.

Discussion

In our screen to identify key components in the LeEIX2-EIX-mediated defence response, we isolated a tomato protein that we call SlRLK-like. LeEIX2 is an RLP but lacks an obvious signalling domain; therefore, an RLK or RLK-like protein is a strong candidate for initiation of its signalling pathway. Although SlRLK-like does not have a consensus kinase domain, it has a malectin-like domain found in all members of the CrRLK1L family (Boisson-Dernier et al. 2011, Galindo-Trigo et al. 2016) that is involved in plant immunity (Boisson-Dernier et al. 2011,

Lindner et al. 2012, Xiang et al. 2008). We demonstrated that this malectin-like domain is essential for the interaction with LeEIX2 and that SlRLK-like is important for regulation of the LeEIX2-EIX response and also associates with the RLK FLS2 and decreases flg22-induced ROS burst.

Over-expression of the SlRLK-like in both *N. benthamiana* and *N. tabacum* resulted in a reduction of all tested defence responses to EIX (Figure 3). Our findings suggest SlRLK-like reduces LeEIX2 protein level, thus negatively regulating the LeEIX2-EIX signalling pathway. Moreover, LeEIX2 is retained in the ER (Figure 2c). Thus, it can be targeted for degradation through an ER-associated degradation process, preventing it from effectively initiating defence responses following EIX application, since it is absent from the plasma membrane where it normally binds EIX (Ron and Avni 2004). The decreased abundance of LeEIX2 is apparent as soon as 24 hpi when co-expressed with SlRLK-like (Supporting information Figure S8). At 48 hpi, when co-expressed with SlRLK-like, LeEIX2 was almost undetectable in cells analysed by confocal microscopy (Figure 6b) and by western blot assay (Figure 6a). Notably, qRT-PCR revealed no significant differences in *LeEIX2* transcript levels with and without SlRLK-like expression, providing additional evidence of regulation at post-translational level (Supporting information Figure S4).

ATG genes and proteins have been shown to crucial for autophagy (Mizushima *et al.* 2011). Interestingly, we found that silencing the *Atg5* gene, inhibited the decrease of LeEIX2 abundance when co-expressed with SlRLK-like (Figure 7). ER-phagy is selective autophagy aimed to restore ER homeostasis when misfolded proteins have accumulated in the ER, for example during unfolded protein response (UPR), and delivering them for degradation (Zeng *et al.* 2019). As we observed LeEIX2 is retained in the ER when co-expressed with SlRLK-like leading to a reduction in its abundance, in addition when autophagy is compromised by silencing of *Atg5*, this reduction is inhibited. Therefore we postulate that LeEIX2 , when co-expressed with SlRLK-like, is being degraded via an ER-phagy process.

Our data suggest that the activity of SlRLK-like resembles that of the mammalian malectin. The mammalian malectin is induced under ER stress conditions and is thought to detect terminally misfolded proteins directly after their expression in the ER lumen (Tannous et al. 2015).

Moreover, overexpression of mammalian malectin in HeLa cells results in enhanced ERAD (Chen *et al.* 2011). Recently, ERAD-II has been described, phenomena where lysosomal, rather than proteasomal, activity appears to result in degradation of ER luminal content, with autophagy possibly being involved (Smith and Wilkinson 2017). The hypothesis that SlRLK-like has properties similar to that of the mammalian malectin is supported by the subcellular localization of SlRLK-like (Figure 2) and our findings that co-expression of different PRRs with SlRLK-like results in a reduction in their protein abundance (Figure 6,Supporting information S5). Altogether, SlRLK-like over-expression leads to a decrease in the available amount of PRRs and attenuated defence responses, which could suggest an additional role for the plant malectin domain, as compared to the mammalian malectin.

Members of the CrRLK1L family take part in a variety of biological processes: cell wall integrity, cellular growth, reproduction, and immunity (Boisson-Dernier et al. 2011, Franck *et al.* 2018). Recent studies found that members of the family are involved in PTI and effector-triggered immunity (Franck et al. 2018). ANXUR1 (ANX1) and ANXUR2 (ANX2) are two members of the *Arabidopsis* CrRLK1L family that are distinct from AT5G24010, the closet *Arabidopsis* homologue of SlRLK-like (Figure 1a). ANX1 and ANX2 attenuate PTI and effector-triggered immunity (Mang *et al.* 2017): *anx1* and *anx2* single-mutant and the *anx1 anx2* double-mutant plants have increased ROS production as well as enhanced MAPK activation in response to flg22 compared to wild-type plants. ANX1 associates with FLS2, BAK1 and BOTRYTIS-INDUCED KINASE1 (BIK1). flg22 elicitation stabilizes the ANX1-BAK1 association, thus interfering with the FLS2-BAK1 complex formation which is essential for FLS2 signalling. In the *anx1* mutant, the enhancement of defence responses was found to be a result of the loss of the ANX1 association with BAK1 (Chinchilla *et al.* 2007). Similarly, in the CRISPR/Cas9 mutated *slrlk-like* tomato lines, elevated ethylene production was observed in response to EIX treatment (Figure 5). The mechanism of negative regulation differs for SlRLK-like and ANX1: SlRLK-like over expression leads to a decrease in available LeEIX2, preventing it from binding to EIX, thus inhibiting a step upstream from the one affected by ANX1.

The function of the plant malectin-like domain has yet to be determined, it has been suggested that this domain is responsible for binding carbohydrates as does the mammalian malectin (Schallus et al. 2008). Specifically, extracellular matrix-derived oligosaccharides have been proposed as ligands for the plant malectin-like domain (Cheung 2011). The weak conservation of the carbohydrate binding residues makes this unlikely (Lindner et al. 2012). However, in this study, we showed that the SlRLK-like malectin-like domain is essential and sufficient for the interaction with and modulation of LeEIX2: The malectin-domain-only truncated version accociate with LeEIX2, whereas the non-malectin tail of the SlRLK-like did not (Figure 4a). The malectin-only domain reduced EIX-induced immune responses to an extent comparable to the full-length SlRLK-like protein (Figure 4b). In addition, we have demonstrated that the malectin-only version is sufficient for causing reduction in LeEIX2 abundance (Figure 6a,b). These data suggest that the malectin-like domain of SlRLK-like acts as a central regulator of LeEIX2 protein levels with the ability to attenuate EIX-induced defence responses.

When different PRRs were co-expressed with SlRLK-like, the effect on protein levels was not uniform. For the RLP AtRLP23 a strong reduction was observed compared to its level when expressed with control mCherry; almost no protein was detected at 24 hpi in the presence of SlRLK-like. For the RLP Ve1 and the RLK FLS2,a moderate yet significant decrease was observed in the presence of SlRLK-like. We confirmed that the association of SlRLK-like with both RLPs Ve1 and RLP23 and the RLK FLS2 *in planta* (Supporting information S7). Moreover, over-expression of SlRLK-like decreased flg22-induced ROS by 50% compared to controls. These findings support our hypothesis that SlRLK-like is a negative modulator of different RLKs and RLPs. The ligands of the tested RLKs and RLPs originate from bacteria and fungi: Ve1 of tomato provides resistance against race 1 strains of *Verticillium dahliae* and *Verticillium albo-atrum* that cause vascular wilt diseases (Fradin *et al.* 2009). AtRLP23 is the RLP for nlp20, the conserved 20-amino-acid fragment found in most necrosis and ethylene-inducing peptide 1-like proteins (Albert et al. 2015). FLS2 is the receptor for bacterial flagellin. Hence, it is plausible that SlRLK-like modulates the protein abundance of both types of PRRs, resulting in indirect negative regulation of immunity signalling.

Evidence of different mechanisms of negative regulation of the LeEIX2 signalling pathway has accumulated in recent years. The *LeEIX* locus of *S. lycopersicum* expresses two receptors: LeEIX1 and LeEIX2. Both receptors bind EIX, but only LeEIX2 is capable of activating downstream signalling (Ron and Avni 2004). LeEIX1 acts as a decoy receptor; it binds EIX and competes with LeEIX2 for EIX binding, thus decreasing EIX signalling (Bar *et al.* 2010). The tomato prenylated RAB acceptor protein 1 directs LeEIX2 to degradation and reduces EIX-induced defence responses (Pizarro *et al.* 2018). Thus, a complex network supervises LeEIX2 protein function, even prior to EIX perception, emphasizing the importance of tightly regulating a signalling pathway able to result in a hypersensitive response and eventually cell death.

Experimental procedures

Plant materials and growth conditions

Nicotiana tabacum cv Samsun NN, *Nicotiana benthamiana*, and *Solanum lycopersicum* cv M82 were grown from seeds in soil (Green Mix; Even-Ari) in a growth chamber, under long-day conditions (16 h:8 h, light: dark) at 24 °C. For stable transformation, tomato seeds were surface sterilized and germinated on Nitsch (Nitsch and Nitsch 1969).

Plasmid construction

For overexpression assays, *LeEIX2* cDNA C-terminally tagged with the sequence coding green fluorescent protein (GFP) was cloned into the *Sal*I site of pBINPLUS (Vanengelen *et al.* 1995) using the following primers: *LeEIX2* forward primer 5′-ATGTCGACATGGGCAAAAGAACTAATC-3′ and *LeEIX2* reverse primer 5′-ATGTCGACGTTCCTTAGCTTTCCCTTCAGTC-3′. *slrlk-like* cDNA C-terminally tagged with sequences encoding GFP, mCherry, or 2xHA was cloned into the *Sal*I *BamHI* sites of pBINPLUS (Vanengelen et al. 1995) using the following primers: *slrlk-like* forward primer 5′-AGGTCGACATGGCGACTCTTTCTTCCACCTTG-3′ and *slrlk-like* reverse primer 5′-ATGGATCCCAGCTTATTAGATGACAGTTG-3′ between the *CAM35SΩ* promoter containing the translation enhancer signal and the *Nos* terminator. The malectin only and non-malectin tail fragments were generated from the full-length plasmid using the following reverse primers and subcloned as described above using reverse primer *slrlk-like malectin only* reverse 5′-ATGGATCCTATCTTCATGATCTCAACCCCATT-3′ and *slrlk-like non-malectin tail* forward primer 5′-GTCGACATGAATAATTCCGTGGGTAGTTTTG-3′.

Yeast two-hybrid screening

Yeast two hybrid screen was done as previously described by Pizarro et al 2018.. For the direct interaction assay, the full-length *slrlk-like* was cloned into prey vector pPR3N using the following primers: *slrlk-like* forward primer 5'-ATTAACAAGGCCATTACGGCCTCCTTTTCACCCCTCGACCAC-3', *slrlk-like* reverse primer 5'-AAATGATTGGCCGAGGCGGCCTCACAGCTTATTAGATGACAGTTG-3'. The plasmid was transformed to NMY51 together with bait vector then plated on media supplemented with 0.1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal).

Phylogenetic tree

Phylogenetic analysis of *Solyc01g094920* was performed using sequences of *CrRLK1L* members from *Arabidopsis thaliana* and *S. lycopersicum*. A maximum likelihood phylogenetic tree was generated with the software http://phylogeny.lirmm.fr/phylo_cgi/phylogeny.cgi using the following pipeline: MUSCLE for multiple alignment, PhyML for tree building, and TreeDyn for tree rendering (Dereeper *et al.* 2008).

Tomato stable transformation

Transgenic *slrlk-like* CRISPR lines were generated using M82 tomato seedlings by cotyledon transformation as previously described (McCormick *et al.* 1986). *Agrobacterium tumefaciens* strain GV3101 harbouring the relevant construct was used for cotyledon co-cultivation. Homozygous *slrlk-like* CRISPR lines *slrlk-like3b-3* and *slrlk-like36b-3* were used for experimental work.

Co-immunoprecipitation

Transient expression was done as previously described by Leibman-Markus *et al.* 2018. CoIP assays were performed essentially as described previously (Leibman-Markus *et al.* 2017). *N. benthamiana* leaves transiently co-expressing LeEIX2-GFP or FLOT1-GFP with SlRLK-like-HA, malectin-only SlRLK-like-HA, or non-malectin tail SlRLK-like-HA were harvested 24 hpi. For CoIP, 500 mg of leaf tissue was used with 13 µl of GFP-TrapA beads (Chromotek).

Bimolecular fluorescence complementation assay

Equal concentrations of Agrobacterium strain GV3101 harbouring plasmids of interest (pSY751-SlRLK-like (YN), pSY752-LeEIX2 (YC)) were transiently co-expressed in *N. benthamiana* leaves and were examined 48 hpi. Negative controls that expressed empty vectors were included in every experiment to verify the specificity of the interactions.

ROS and Ethylene measurements

ROS burst and Ethylene mesurments were measured as previously described (Leibman-Markus et al. 2017).

Hypersensitive response detection

N. tabacum plants were infiltrated with a mixture of *A. tumefaciens* harbouring EIX expression vector (OD_{600}=0.03) and SlRLK-like-HA expression vector (OD_{600}=0.2) or empty vector (OD_{600}=0.2). HR was monitored over a period of 48 h. Photographs were taken every 30 min starting at 12 hpi to track HR progression.

RNA extraction and qRT-PCR analysis

Plant total RNA was extracted using SV Total RNA Isolation System (Promega). RNA samples (4 µg) were used for first-strand cDNA synthesis using M-MLV reverse transcriptase (Promega) and oligo(dT_{15}). qRT-PCR was performed according to the Fast SYBR Green Master Mix protocol (Life Technologies/Thermo Fisher) using a StepOnePlus Real Time PCR System (Thermo Fisher). *LeEIX2* (*Solyc07g008630*) expression in *N. benthamiana* co-expressing *LeEIX2* and *slrlk-like* or control was examined using forward primer 5′-ACCAGGAGTCCGAGTACAAGA-3′ and reverse primer 5′-TGACAAGTCGAGGGACTCCA-3′. *Atg5* expression in VIGS-silenced *N. benthamiana* was examined using forward primer 5'- CCGGAGCTGTTTGGGGAAAA -3' and reverse primer 5'- CTCTCCACTTTGTCCCGTGC-3'. Endogenous normalizing gene *NbUbiquitin* (*TC20187*) expression was examined using forward primer 5'-AATGTGAAAGCCAAGATCCAAG-3' and reverse primer 5'-CGGAGGCGGAGCACGAGATGAA-3' (Liu *et al.* 2012). *SlACS2* (*Solyc01g095080*) expression

in *slrlk-like* CRISPRed lines was examined using forward primer 5′-TCACCATACTACCCAGCATTTAACA-3′ and reverse primer 5′-TGGAGCTCTCACAGTGAATTGG-3′. Endogenous normalizing gene *SlCyclophilin* (*Solyc01g111170*) was amplified using forward primer 5′-TGAGTGGCTCAACGGAAAGC-3′ and reverse primer 5′-CCAACAGCCTCTGCCTTCTTA-3′. qRT-PCR was performed using the following program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Control samples without reverse transcriptase did not generate a PCR product after 40 amplification cycles indicating that the samples were free of genomic DNA contamination.

Western blot

Western blot was performed on *N. benthamiana* leaves transiently expressing relevant constructs as previously described (Leibman-Markus et al. 2017).

Confocal microscopy

Confocal microscopy images were acquired as previously described by Leibman-Markus et al. 2018.

CRISPR-Cas9 genome editing

CRISPR-Cas9 editing was performed in tomato using a previously described procedure (Fauser *et al.* 2014). A small guide RNA targeting *slrlk-like* was designed to include an *AvaII* restriction site three nucleotides upstream of the PAM site to enable screening of editing events (sgRNA *slrlk-like* forward 5'- ATTGCCTCGTAAACTGTGGGTCCC-3' and reverse 5'-AAACGGGACCCACAGTTTACGAGG-3'). The sgRNA was also designed to have sticky ends compatible with *Bsa*I sticky ends (indicated in lower case letters) and was subcloned into digested pEN-Chimera combined with pCAS9-TPC (Fauser et al. 2014) and termed pCAS9-Chimera. The *A. tumefaciens* strain GV3101 harbouring pCAS9-Chimera with *slrlk-like* sgRNA was used for *S. lycopersicum* cv M82 transformation. Total DNA was extracted from transformed plants and used as a template for *slrlk-like* sgRNA flanking-fragment amplification. PCR fragments were digested with *AvaII* and completely uncut fragments were presumed to originate from plants edited in both alleles, which was verified by sequencing. Relevant transgenic lines were self-pollinated, and the resulting T1 plants were re-analysed. Four or five leaves from 6-week-old plants were used for physiological assays.

Virus-induced gene silencing

VIGS was performed in tomato according to Liu, Schiff, and Dinesh-Kumar (2002). *A. tumefaciens* strain GV3101 harbouring TRV RNA1 (pYL155) and TRV RNA2 (pYL170) was mixed in a 1:1 ratio in infiltration buffer. TRV RNA2 empty served as a control and a specific primer of NbATG5 (Niben101Scf01320g03007.1) was cloned into TRV RNA2 specifically targeting the *Atg5* gene. Agrobacterium mixtures were infiltrated into cotyledons of 5-day-old tomato *N. benthamiana* seedlings. Six-week-old plants were used for gene-level expression measurements and physiological assays. Three plants resulting from independent silencing events were used in each experiment.

Accession numbers

A0A3Q7F5H9 (SlRLK-like)**,** Q9FLW0 (CrRLK)**,** Q6JN46 (LeEIX2)**,** P18485 **(SlACS),** P21568 **(SlCyclophylin)**

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Author Contributions

O.S, S.S and L.P carried out the experiments. O.S and A.A wrote the manuscript. No conflict of interest declared.

Conflict of Interest

The authors have no conflict of interest to declare

Data Availability Statement

All relevant data can be found within the manuscript and its supporting materials

Supporting information

Additional Supporting information may be found in the online version of this article.

Figure S1 NMY51 yeast cells expressing SlRLK-like (in pPR3-N) together with empty vector, FLOT1 or LeEIX2 (in pBT3-SUC) were grown on auxotrophic media (-Trp, -Leu, -His, -Ade) and tested for activation of the reporter gene *LacZ* by plating on auxotrophic media (-Trp, -Leu) supplied with X-gal

Figure S2 *N. tabacum* leaves were transiently transformed with non-malectin-tail-HA or FLOT1-mCherry (control) and a) ethylene biosynthesis and b) ROS burst was quantified in samples following EIX treatment. Ethylene biosynthesis levels are expressed as a percentage of the control sample in the presence of EIX. Values are the means and standard error of three independent experiments, N_{total}=15; t-test, no significant difference was detected. Luminescence expressed by relative light units (RLU) was measured immediately following EIX treatment to track the ROS burst. Values are the means and standard error of three independent experiments, n=12 each; **p-value<0.001 *p-value<0.05, two-way analysis of variance

Figure S3 Amino acid sequences of SlRLK-like in M-82 (WT) tomato and in the stable lines created using CRISPR/Cas9

Figure S4 qRT-PCR quantification of *LeEIX2* expression in *N. benthamiana* co-expressing LeEIX2-GFP together with SlRLK-like-mCherry or free mCherry (control). Expression was normalized to *NbUbiquitin*, N_{total}=9, t-test. The difference was not significant

Figure S5 Co-expression of SlRLK-like with PRRs leads to a reduction in their protein abundance. SlRLK-like-mCherry or mCherry (control) were co-expressed in N. benthamiana with

PRRs (a) Ve1-GFP, (b) RLP23-GFP, (c) FLS2-GFP, (d) FLOT1-GFP. Protein levels were detected by SDS-PAGE and western blot. The experiments were replicated three times independently, representative images are shown.

Figure S6 Pixel intensity of (a) SlRLK-like and (b) FLOT1 as measured in confocal images of *N. benthamiana* ATG5-silenced (VIGS-ATG5) or control (VIGS-Empty), N_{total}=14, t-test. The difference was not significant.

Figure S7 *N. benthamiana* was transiently co-transformed with SlRLK-like-HA together with RLP23-GFP, Ve1-GFP or FLOT1-GFP. Total protein (input) and immunopurified proteins bound to GFP-beads (IP) were separated on SDS-PAGE followed by western blot using anti-HA antibody for SlRLK-like detection and anti-GFP antibody for RLP23-GFP, Ve1-GFP or FLOT1-GFP detection

Figure S8 *N. benthamiana* transiently co-transformed with LeEIX2-GFP and SlRLK-like-mCherry or Free mCherry (control) at 24 and 48 hpi were analysed by SDS-PAGE and western blot

Table S1 Clones identified in the yeast two-hybrid screen

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Figure legends

FIGURE 1 Solyc01g094920, which we named SlRLK-like, is an interactor of LeEIX2. (a) Maximum likelihood phylogenetic tree of *Solyc01g094920* created using DNA sequences of *CrRLK1L* genes from *Arabidopsis thaliana* and *Solanum lycopersicum*. Solyc01g094920 is marked in blue. (b) Schematic representation of SlRLK-like and its close homolog from *Arabidopsis* (At5g24010). SP indicates signal peptide, TM indicates the transmembrane domain. (c) NMY51 yeast cells expressing LeEIX2 (in pBT3-SUC) together with control (empty vector), SlRLK-like C-terminal region, or full-length SlRLK-like (in pPR3-N) were grown on auxotrophic media (-Trp, -Leu, -His, -Ade) and tested for activation of the reporter gene *LacZ* by plating on auxotrophic media (-Trp, -Leu) supplied with X-gal.

FIGURE 2 SlRLK-like interacts with LeEIX2 in plants. (a) *N. benthamiana* was transiently co-transformed for expression of SlRLK-like-HA with LeEIX2-GFP or FLOT1-GFP (control). Total protein (input) and immunopurified proteins bound to GFP-beads (IP) were separated on SDS-PAGE followed by western blot using anti-HA antibody for SlRLK-like detection and anti-GFP antibody for LeEIX2 and FLOT1 detection. (b) Confocal microscopy image of *N. benthamiana* epidermal cells transiently co-expressing YC-YFP alone or fused to LeEIX2 or FLOT1 and YN-YFP fused to SlRLK-like. White arrowhead points to nuclear envelope and square marks ER-like structures Scale bar, 20 µm. In box: detection of FLOT1-YC using anti-HA antibody (c) Confocal microscopy images of *N. benthamiana* epidermal cells transiently co-expressing SlRLK-like-mCherry with HDEL-mCherry or FLOT1-mCherry showing SlRLK-like effect over LeEIX2 subcellular localization. Scale bar 10 µm. (d) Confocal microscopy images of *N. benthamiana* epidermal cells transiently co-expressing LeEIX2-GFP with FLOT1-mCherry, SlRLK-like-mCherry, HDEL-mCherry or FLS2-mCherry showing SlRLK-like localization. Scale bar, 10 µm. White arrowheads points to co-localization between SlRLK-like and LeEIX2 in ER.

FIGURE 3 SlRLK-like expression decreases EIX-mediated defence responses (a-b) *N. tabacum* leaves were transiently transformed with SlRLK-like-HA or NOS pro::GFP (control) and a) ethylene biosynthesis and b) ROS burst were quantified in samples following EIX treatment. Ethylene biosynthesis levels are expressed as percentage of the control sample in the presence of EIX. Values are the means and standard error of three independent experiments, N_{total}=18 ; p-value<0.0001, t-test. Luminescence expressed by relative light units (RLU) was measured immediately following EIX treatment to track the ROS burst. Values are the means and standard error of three independent experiments, n=12 each; ***p-value<0.0001 **p-value<0.001 *p-

value<0.05, two-way analysis of variance. (c) HR development in *N. tabacum* leaves transiently transformed with a mixture of *A. tumefaciens* harbouring EIX and SlRLK-like or empty vector at time points indicated.

FIGURE 4 The malectin region of SlRLK-like interacts with LeEIX2. (a) *N. benthamiana* transiently co-transformed with malectin-only-HA or non-malectin tail-HA together with LeEIX2-GFP or FLOT1-GFP. Total protein (input) and immunopurified proteins bound to GFP-beads (IP) were loaded onto SDS-PAGE followed by western blot using anti-HA antibody for malectin-only and non-malectin tail detection and anti-GFP antibody for LeEIX2 or FLOT1 detection. (b-c) *N. tabacum* leaves were transiently transformed with malectin-only-HA or NOS pro::GFP (control) then b) ethylene biosynthesis and c) ROS burst were measured in samples following EIX treatment. Ethylene biosynthesis levels are expressed as percentage of the control sample in the presence of EIX. Values are the means and standard error of three independent experiments, N_{total}=15 ; p-value<0.0001, t-test. Luminescence expressed by relative light units (RLU) was measured immediately following EIX treatment to track the ROS burst. Values are the means and standard error of three independent experiments, n=12 each; ***p-value<0.0001 **p-value<0.001 *p-value<0.05, two-way analysis of variance.

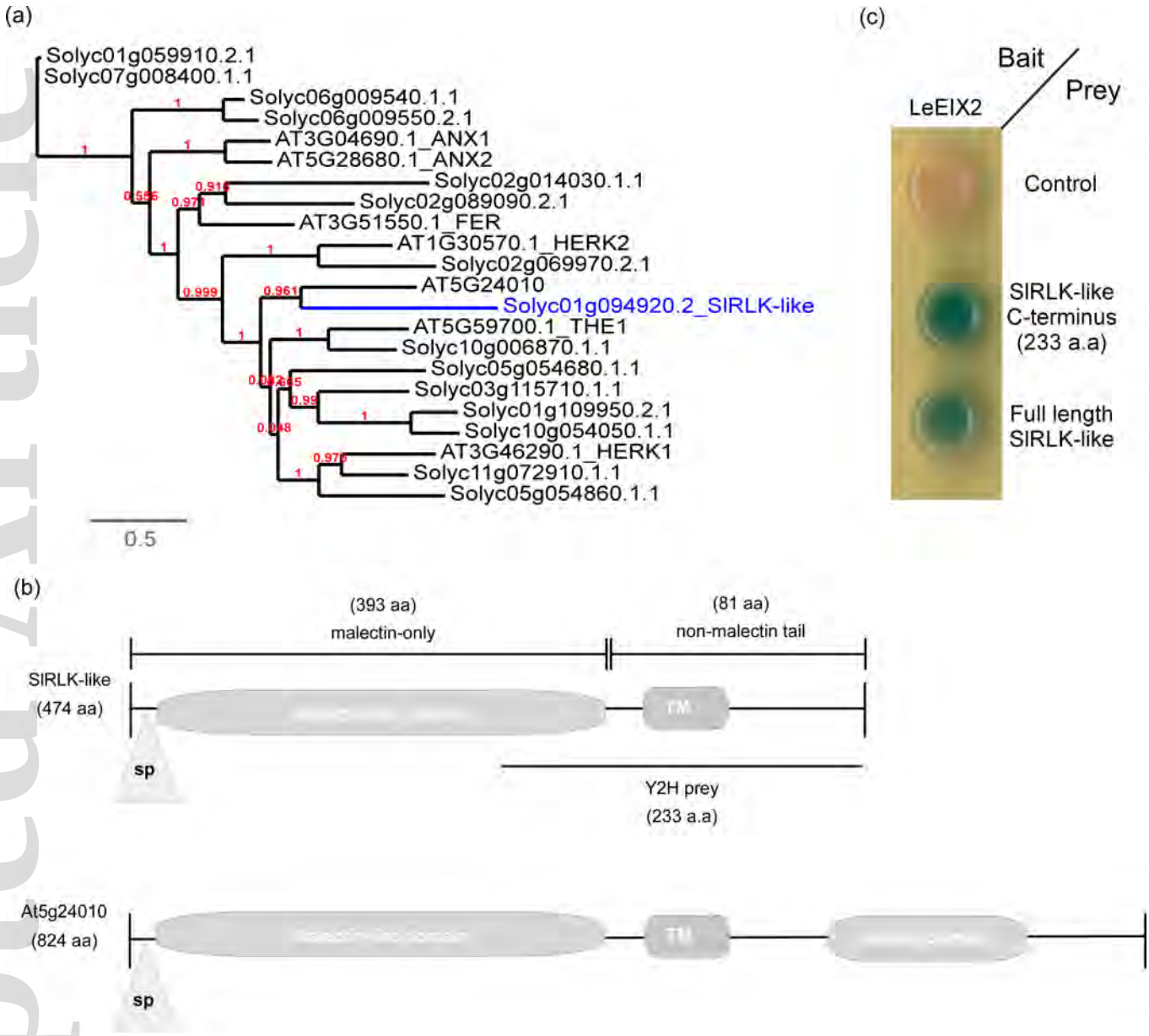
FIGURE 5 Loss of SlRLK-like results in elevated production of ethylene in response to EIX. Stable tomato lines with deletions in *SlRLK-like* or unedited control were subjected to (a) measurements of ethylene biosynthesis and (b) qRT-PCR for *SlACS2* expression following EIX application. Ethylene biosynthesis levels are expressed as percentage of the control sample in the presence of EIX. Values are the means and standard error (SE) of three experiments, N_{total}=14; ***p-value < 0.0001, t-test. *SlACS* expression was normalized to expression of *SlCyclophilin*; N_{total}=9 **p-value<0.001, ***p-value<0.0001 one-way analysis of variance.

FIGURE 6 Co-expression with SlRLK-like reduces levels of LeEIX2. Extracts of *N. benthamiana* transiently co-transformed with LeEIX2-GFP and either SlRLK-like-mCherry, malectin-only, non-malectin tail or free mCherry (control) were analyzed by (a) SDS-PAGE and western blot (b) confocal microscopy at 48 hpi (scale bar, 20 µm). (c) LeEIX2-GFP expression level measured by pixel intensity when co-expressed with SlRLK-like-mCherry and either SlRLK-like-mCherry, malectin-only, non-malectin tail or free mCherry (control), N_{total}=24; p-value<0.0001, t-test

FIGURE 7 Silencing of *NbATG5* leads to a reduced SlRLK-like-mediated LeEIX2 abundance

reduction. (a) qRT-PCR quantification of *NbATG5* expression in *N. benthamiana*. Expression was normalized to *NbUbiquitin*. Extracts of *N. benthamiana* epidermal cells when transiently co-expressed with SlRLK-like (b,d) or FLOT1 (c,e) in ATG5-silenced (VIGS-ATG5) or control (VIGS-Empty) were analyzed by (d-e) confocal microscopy at 48 hpi (scale bar, 10 µm) ;(b-c) pixel intensity, N_{total}=14, p-value<0.001, one-way ANOVA and Tukey post-tests; and (f) SDS-PAGE and western blot.

FIGURE 8 The complex of SlRLK-like and FLS2 attenuates flg22-induced ROS. *N. tabacum* leaves were transiently transformed with SlRLK-like-HA together with FLS2-GFP or FLOT1-GFP. Total protein (input) and immunopurified proteins bound to GFP-beads (IP) were loaded onto SDS-PAGE followed by western blot using anti-HA antibody for SlRLK-like-HA detection and anti-GFP antibody for FLS2 or FLOT1 detection. (b) *N. tabacum* leaves transiently transformed with SlRLK-like-HA or NOSpro::GFP (control) were subjected to measurements of ROS burst following flg22 application. Luminescence, expressed in RLU, was measured immediately following flg22 application to track the ROS burst. Values are the means and standard error of three independent experiments, n=12 each; ***p-value<0.0001 **p-value<0.001, two-way analysis of variance.



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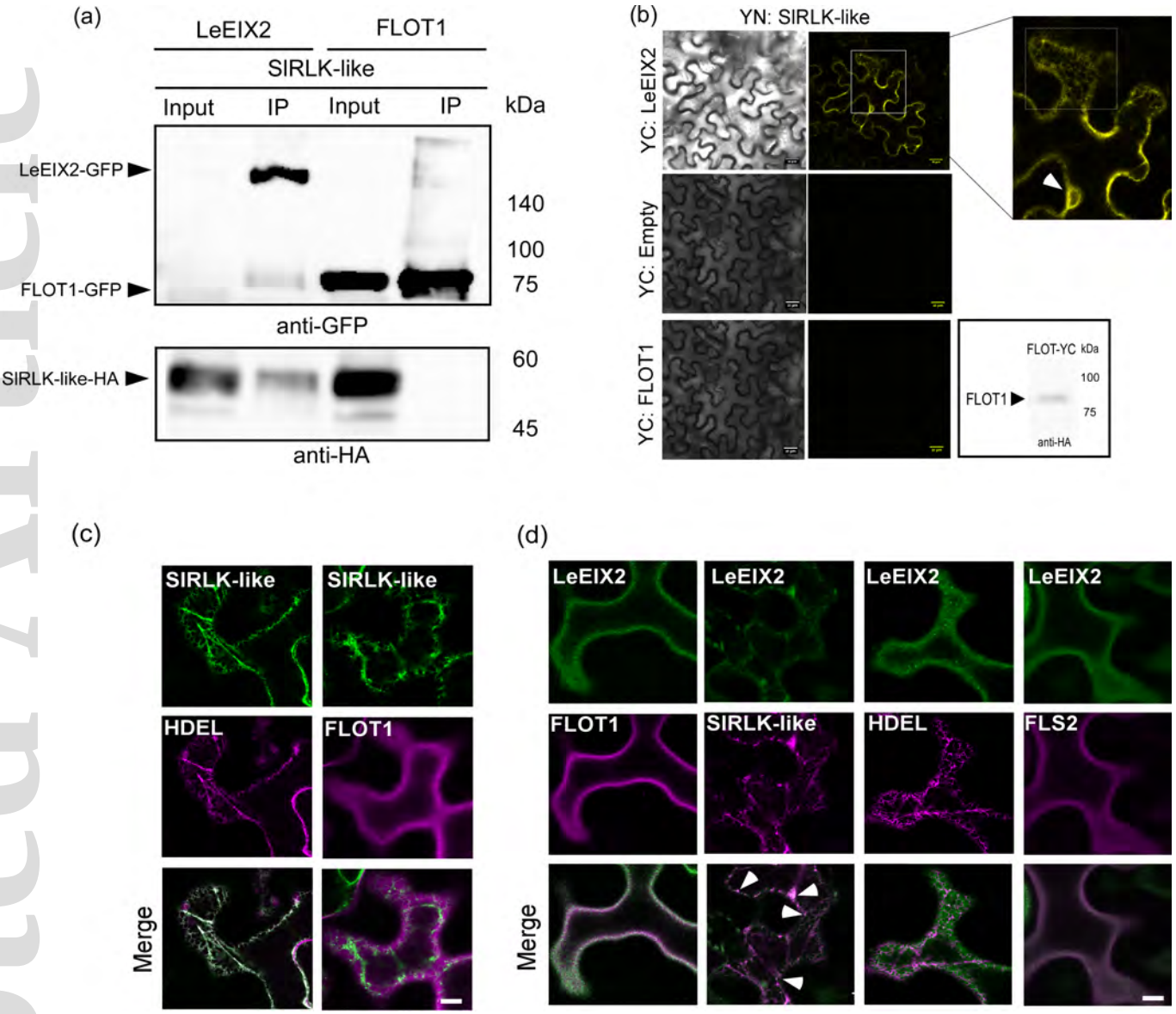

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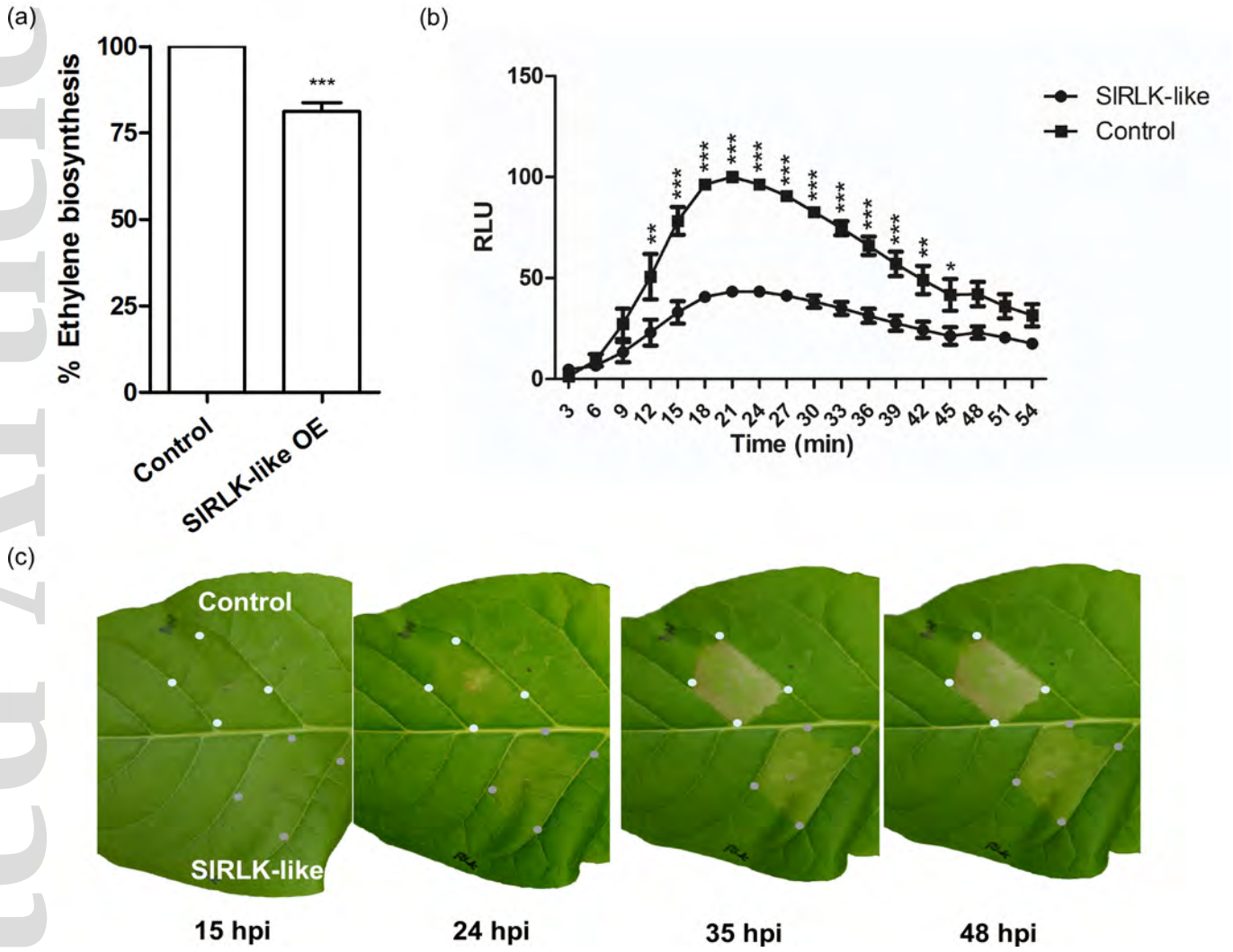

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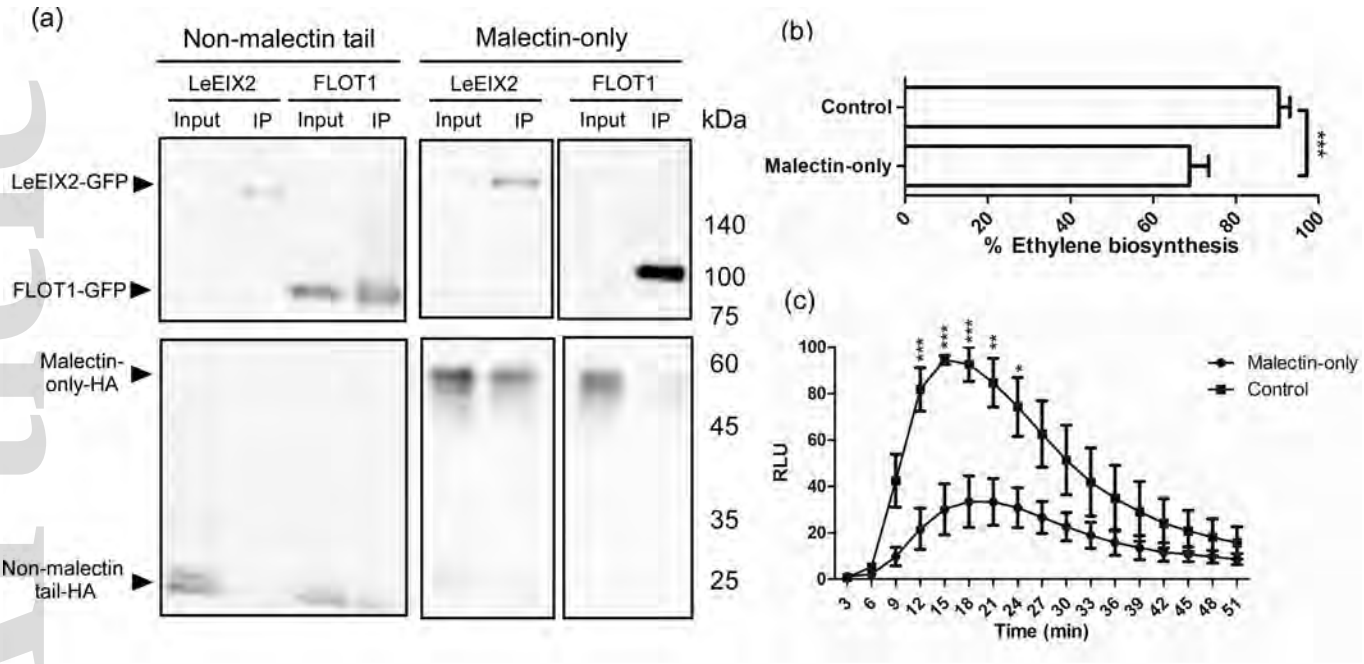

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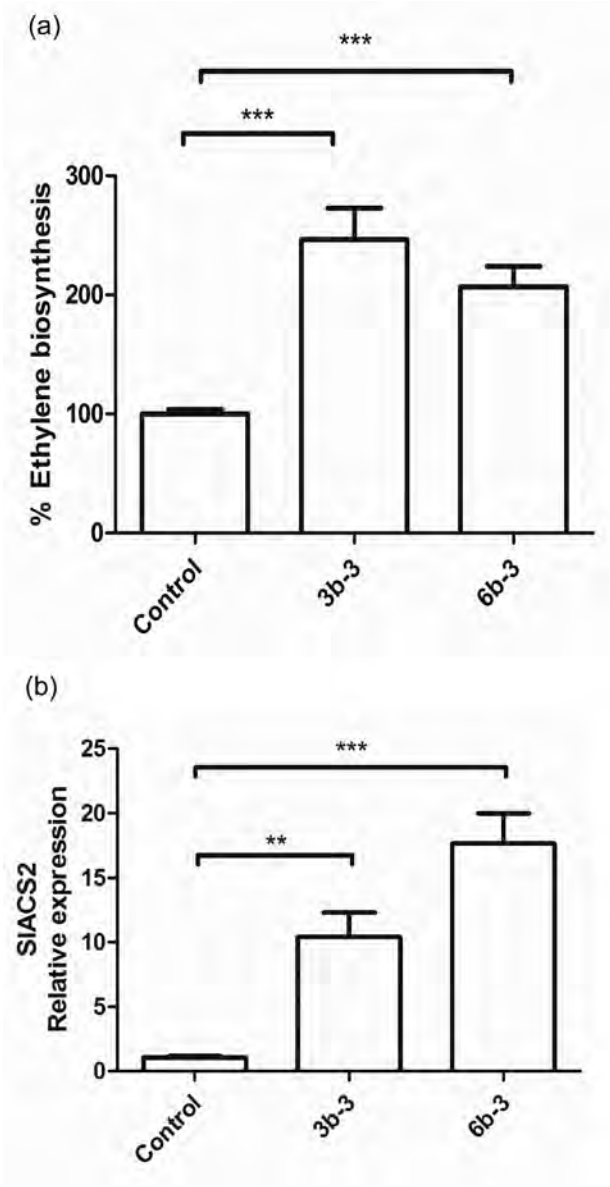

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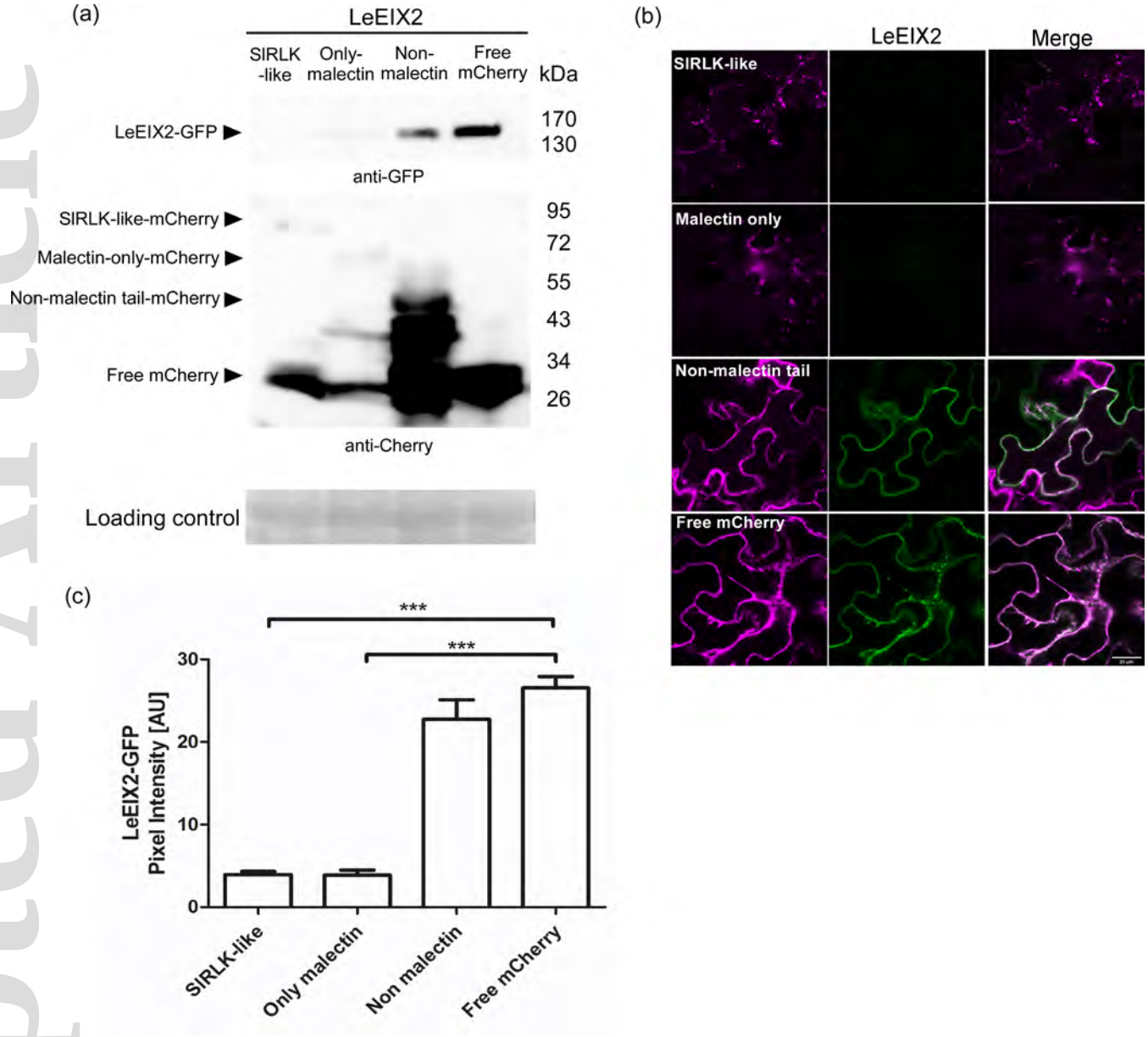

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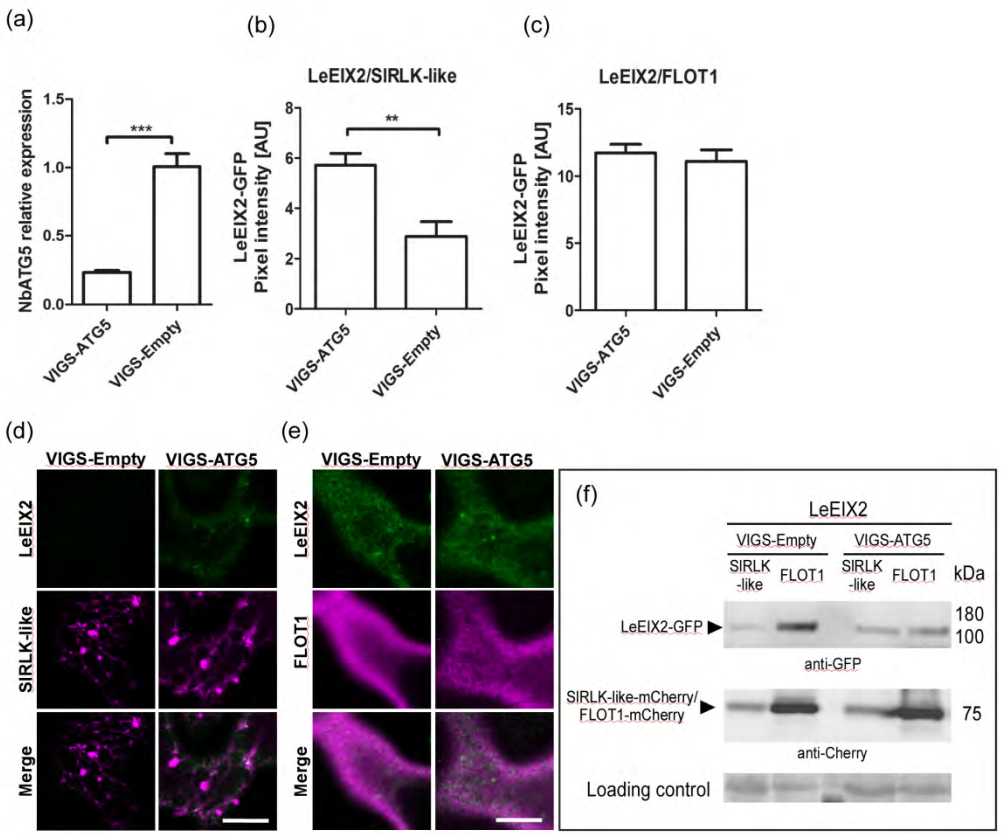

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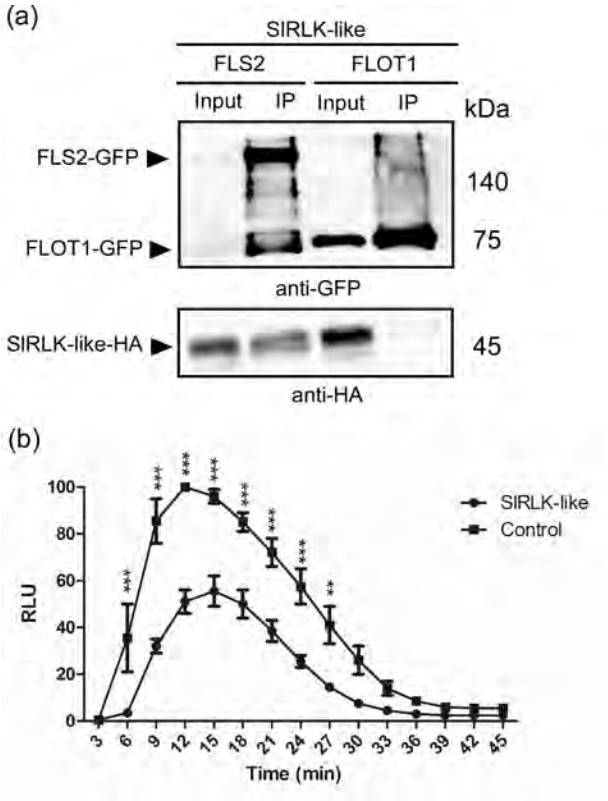

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