LORE homomerization is required for 3-OH-C10:0 induced immune signaling

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ABSTRACT
Perception and processing of various internal and external signals is essential for all living organisms. Plants have an expanded and diversified repertoire of cell surface-localized receptor-like kinases (RLKs) that transduce signals across the plasma membrane. RLKs often assemble into higher-order receptor complexes with co-receptors, regulators and scaffolds to convert extracellular stimuli into cellular responses. To date, the only S-domain-RLK from Arabidopsis thaliana with a known ligand and function is AtLORE, a pattern recognition receptor that senses bacterial 3-hydroxy fatty acids of medium chain length, such as 3-hydroxy decanoic acid (3-OH-C10:0), to activate pattern-triggered immunity. Here we show that AtLORE forms receptor homomers, which is essential for 3-OH-C10:0-induced immune signaling. AtLORE homomerization is mediated by the transmembrane and extracellular domain. We show natural variation in the perception of 3-OH-C10:0 within the Brassicaceae family. Arabidopsis lyrata and Arabidopsis halleri do not respond to 3-OH-C10:0, although they possess a putative LORE orthologue. We found that LORE orthologues of these 3-OH-C10:0 nonresponsive species have defective extracellular domains that can bind the 3-OH-C10:0 ligand but lack the ability to homomerize. Our findings shed light on the activation mechanisms of AtLORE and explain natural variation of 3-OH-C10:0 perception within the Brassicaceae family.

KEYWORDS
Receptor-like kinase, S-domain, LORE, homomerization, pattern recognition receptor, pattern-triggered immunity, microbe-associated molecular pattern
INTRODUCTION

Perception, processing and integration of a wide range of environmental and cellular stimuli is fundamental to all living organisms. In plants, this is implemented, amongst others, by members of the super-families of receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Shiu and Bleecker, 2001, 2003; Hohmann et al., 2017; Jamieson et al., 2018; Dievart et al., 2020). They regulate various cellular processes such as growth, development, reproduction or immunity (Shiu and Bleecker, 2001; De Smet et al., 2009; Li and Yang, 2016; Boutrot and Zipfel, 2017; Gou and Li, 2020). Most RLKs comprise an extracellular, presumably ligand binding domain (ECD) with a variety of sequence motifs, a single-span transmembrane domain (TMD) and an intracellular domain (ICD) comprising a serine/threonine protein kinase domain for signal transduction (Shiu and Bleecker, 2001; Dievart et al., 2020). In many cases, additional tyrosine phosphorylation activity was observed. Presumably, RLKs are generally dual-specificity kinases, (Bojar et al., 2014; Macho et al., 2015), but pseudo kinases also exist. RLPs, lacking an intracellular kinase domain, are incapable of signal transduction on their own and require signaling competent partners (Jamieson et al., 2018). RLKs and RLPs are classified according to their ECD motifs, such as leucine-rich-repeat (LRR), L-type lectin (Lec), S-domain (SD) or lysin-motif (LysM) domains (Shiu and Bleecker, 2001, 2003; Dievart et al., 2020).

A common theme of RLK and RLP signaling is their association into higher order receptor complexes via homo- or hetero-oligomerization. They can act as ligand-binding receptors, coreceptors, scaffolds or positive/negative regulators to orchestrate and fine tune signaling (Ma et al., 2016b; Burkart and Stahl, 2017; Wan et al., 2019; Gou and Li, 2020). To date, we have gained a detailed molecular and structural understanding of ligand binding, activation mechanisms and the role of receptor complex formation for a few RLKs, which have become important receptor models. One prototypical heteromeric signaling complex is the heterodimer between LRR-RLK FLAGELLIN SENSING 2 (AtFLS2) and its LRR-co-receptors of the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (AtSERK) family, particularly BRI1-ASSOCIATED RECEPTOR KINASE 1/SEMK3 (AtBAK1) from A. thaliana (Gomez-Gomez and Boller, 2000; Chinchilla et al., 2006; Chinchilla et al., 2007; Roux et al., 2011). FLS2 functions as pattern recognition receptor (PRR) in plant immunity. PRRs perceive microbe-associated molecular patterns (MAMPs) to induce pattern-triggered immunity (PTI) (Boutrot and Zipfel, 2017). Perception of the ligand, the peptide epitope flg22 of bacterial flagellin, triggers association of the AtFLS2 receptor with its co-receptors (Boller and Felix, 2009; Robatzek and Wirthmueller, 2013; Couto and Zipfel, 2016). Co-crystallization of the ECDs of both AtFLS2 and AtBAK1 with flg22 shows a series of intermolecular bonds that stabilize the heterodimer in both ligand- and receptor mediated manner (Sun et al., 2013b). State of the art imaging techniques enable real-time analysis of receptor dynamics in vivo and suggested that AtFLS2/AtBAK1 heterodimers can further associate into tetrameric complexes (Somssich et al., 2015). The multicomponent PRR complex for perception of the fungal MAMP chitin is characterized in detail as well. In Arabidopsis, three LysM-RLKs (LYK), AtLYK5, AtLYK4 and the CHITIN ELICITOR RECEPTOR KINASE 1 (AtCERK1/ AtLYK1) cooperate in chitin perception and signaling (Miya et al., 2007; Cao et al., 2014; Xue et al., 2019; Gong et al., 2020). Upon chitin binding, the pseudo-kinase AtLYK5 hetero-dimerizes with AtCERK1, triggering further homomerization of AtCERK1. This leads to the formation of a sandwich-like receptor complex to enable downstream signaling (Couto and Zipfel, 2016; Gong et al., 2020). AtLYK4 interacts with both AtLYK5 and AtCERK1 and was reported to have a role in scaffolding or ligand binding (Gong et al., 2020).
While ligand induced hetero-dimerization with co-receptors is essential for signaling in the previous examples, the mode of action is different for S-LOCUS RECEPTOR KINASEs (SRKs) from *Brassica rapa*. *BraSRKs* belong to the family of S-domain-RLKs (SD-RLKs, also referred to as G- or B-type lectin RLKs) which comprises about 40 members in Arabidopsis and more than 100 in rice (Shiu and Bleecker, 2001; Shiu et al., 2004; Vaid et al., 2012; Xing et al., 2013; Teixeira et al., 2018). SRKs and their ligands, namely S-LOCUS CYSTEIN-RICH PEPTIDE (SCRs), mediate self-incompatibility (SI) to maintain genetic variability by avoiding inbreeding (Ivanov et al., 2010; Nasrallah and Nasrallah, 2014; Jany et al., 2019). Recognition of pollen-secreted SCRs by SRKs expressed in the stigma enables the plant to detect and subsequently reject self-pollen (Nasrallah and Nasrallah, 2014; Jany et al., 2019). The ECD of SRKs comprises two G-type lectin-like, an epidermal-growth factor (EGF)-like and a plasminogen-apple-nematode (PAN) domain, representing one typical domain architecture of SD-RLKs (Shiu and Bleecker, 2001; Xing et al., 2013; Dievart et al., 2020). Spontaneous and ligand-independent homomerization of SRKs via the ECD has been reported (Giranton et al., 2000; Naithani et al., 2007). However, the crystal structures obtained from *BraSRK9* and engineered *BraSRK8* elucidated, that two SKRs and two of the respective SCR peptides form a 2:2 hetero-tetrameric complex in a ligand- and receptor-mediated manner (Ma et al., 2016a; Murase et al., 2020). Receptor homo-dimerization of SRKs appears to be essential for SRK signaling. To date, no co-receptors are found to be involved in SRK receptor complexes.

Apart from SRKs, only a few SD-RLKs from different plant species have been described in more detail. They are involved in plant-pathogen interactions, symbiosis or abiotic stress responses (Navarro-Gochicoa et al., 2003; Chen et al., 2006; Kanzaki et al., 2008; Kim et al., 2009a; Kim et al., 2009b; Kim et al., 2015; Chen et al., 2013; Cheng et al., 2013; Sun et al., 2013a; Zou et al., 2015; Fan et al., 2018; Schnepf et al., 2018; Labbé et al., 2019; Jinjun et al., 2020; Pan et al., 2020; Sun et al., 2020; Liu et al., 2021; Zhou et al., 2021). In *Glycine soja*, S-LOCUS LecRK (GsSRK) regulates osmotic homeostasis and plant architecture under salt stress (Sun et al., 2013a; Sun et al., 2018). *Oryza sativa* OsPi-d2 confers resistance to rice blast caused by the fungus *Magnaporthe grisea* (Chen et al., 2006). Interestingly, natural variation in a single amino acid position in the TMD appears to define resistant or susceptible alleles (Chen et al., 2006; Li et al., 2015). Expression of the LARGE SPIKE S-DOMAIN RECEPTOR LIKE KINASE 1 (OsLSK1) in rice is regulated by growth hormones and is associated with abiotic stress sensitivity and yield. The ECD of OsLSK1 interacts with itself and heteromerizes with the ECDs of five homologous SD-RLKs. Overexpression of truncated OsLSK1 lacking the ICD increases plant height and grain yield, supposedly by exerting a dominant negative effect on endogenous OsLSK1 signaling (Zou et al., 2015). Although a growing number of members of the large SD-RLK sub-family are being characterized, their ligands, receptor complex formation and downstream signaling mechanisms remain largely unknown. Biochemical characterization of SD-RLKs and identification of ligands is proving to be particularly challenging. Large-scale expression and purification of SD-RLKs appears to be especially difficult, likely because of their complex folding, high degree of glycosylation, and tendency of protein aggregation (Murase et al., 2020; Sun et al., 2020).

In *A. thaliana*, we previously discovered the SD-RLK AtLORE (LIPOOLIGOSACCHARIDE SPECIFIC REDUCED ELICITATION or SD1-29) as PRR for bacterial medium-chain 3-hydroxylated fatty acid (3-OH-FA) metabolites (Ranf et al., 2015; Kutschera et al., 2019). 3-OH-FAs are sensed in a chain-length and hydroxylation specific manner (Kutschera et al., 2019). Only 3-OH-FAs with acyl chains comprising 8 – 12 carbon atoms activate AtLORE signaling, with 3-hydroxydecanoic acid (3-OH-C10:0) being the strongest elicitor in *A. thaliana*. 3-OH-C10:0 directly binds to the AtLORE ECD, as evidenced by microscale thermophoresis (MST) and ligand
depletion-binding assays (Kutschera et al., 2019; Shu et al., 2021). AtLORE is the first SD-RLK in
A. thaliana with a known ligand and thus a controllable means of activation, making it an important
model for mechanistic studies of the SD-RLK family. Recently, downstream signaling components
of AtLORE were elucidated. Sensing of 3-OH-C10:0 leads to phosphorylation of AtLORE at Y600,
which is required for phosphorylation and activation of the receptor-like cytoplasmic kinase PBS1-
LIKE 34 (AtPBL34). AtLORE directly interacts with AtPBL34 and its homologs AtPBL35 and
AtPBL36, which seem to redundantly facilitate downstream signaling in AtLORE-mediated
immune responses (Luo et al., 2020). To date, no co-receptor has been found to be involved in
AtLORE signaling and the steps of receptor complex formation are unknown.

Here, we show that AtLORE forms receptor homomers via TMD and ECD, which is required for
3-OH-C10:0-induced immune signaling. Interestingly, we found natural variations in
responsiveness to 3-OH-C10:0 in some closely related Brassicaceae, which have LORE
orthologues with high protein sequence identity to AtLORE. Functional analysis of these
orthologues and chimera with AtLORE shows that orthologues from nonresponsive species have
defects in their ECDs. Interestingly, the defective LORE orthologues still bind the 3-OH-C10:0
ligand via their ECDs but are compromised in homomerization. This highlights that receptor
homomerization is essential for LORE-dependent immune signaling. Moreover, our results
indicate that in the case of LORE, ligand binding is independent of homomerization, in contrast to
ligand- and receptor-mediated homodimerization of BraSRKs. Our results show that two
Brassicaceae species closely related to A. thaliana lack the ability to respond to 3-OH-C10:0,
which is explained by altered homomerization ability. This sheds light on the mechanistic aspects
of 3-OH-C10:0 sensing in Brassicaceae.

RESULTS

AtLORE forms receptor homomers in planta

We performed multiple sequence alignments (MAFFT algorithm, Jalview) (Fig. S1) of the amino
acid sequence of the ECDs of AtLORE (AT1G61380) and well-studied SRKs from Brassica rapa,
BraSRK9 (BAA21132.1) and BraSRK8 (BAF91375). Labeling of percentage identity shows a high
sequence similarity and conservation of disulfide bridge forming cysteine residues in the EGF and
PAN domain, indicating an overall similar domain architecture of AtLORE and BraSRKs. Transient
agrobacterium-mediated overexpression of kinase-active AtLORE in Nicotiana benthamiana,
which lacks a putative LORE orthologue (Ranf et al., 2015), results in a cell death-like necrotic
phenotype. These cell death symptoms can be visualized by trypan blue staining (Fig. S2E) and
an increased chlorophyll fluorescence (Fig. 1A), which is associated with cell death (Landeo
Villanueva et al., 2021). This phenomenon is not observed upon expression of AtLORE-Km, which
is mutated at the conserved ATP-binding site (K516A) in the kinase domain and has been shown
to be signaling incompetent (Ranf et al., 2015). Therefore, we assume that cell death is caused
by spontaneous receptor activation. Based on the high protein sequence similarity of AtLORE to
BraSRKs and the receptor auto-activation of AtLORE upon transient overexpression in N.
benthamiana, we hypothesized that AtLORE may form homomers like BraSRKs. Indeed, AtLORE
homomerizes in vivo upon transient expression in N. benthamiana. GFP and mCherry fusion
proteins of kinase-active and kinase-mutated (Km, K516A) AtLORE co-immunoprecipitate (Co-IP)
with each other (Fig. 1B). AtLORE homomerization was also observed in bimolecular
fluorescence complementation (BiFC) assays using AtLORE-split-YFP N-/C-terminal fragment
expression (SPYCE/SPYNE) fusion proteins. To avoid background auto-fluorescence caused by
the cell-death symptoms, AtLORE-Km was used for fluorescence-based assays. Transient co-expression of AtLORE-Km fused to either SPYCE or SPYNE restores YFP fluorescence, detectable via laser scanning microscopy and fluorescence quantification (Fig. S2A-C). We performed Förster resonance energy transfer (FRET) fluorescence lifetime imaging (FLIM) to proof direct physical LORE-LORE interaction (Fig. 1C and Fig. S2D). Co-expression of AtLORE-Km-GFP and AtLORE-Km-mCherry results in a significant reduction in GFP fluorescence lifetime compared to the FRET-donor only control AtLORE-Km-GFP. mCherry-tagged Glutathione-S-transferase of A. thaliana (AT1G17170, AigST-mCherry), was used as co-expression control and does not reduce GFP fluorescence lifetimes. An AtLORE-Km-GFP-mCherry fusion protein serves as FRET positive control. AtLORE homomerization is not influenced by the presence of its ligand 3-OH-C10:0 in FRET-FLIM experiments (Fig. 1D). Thus, under the experimental conditions used, AtLORE forms homomers in the plasma membrane in a ligand-independent manner.

AtLORE homomerization is mediated by the extracellular- and transmembrane domain

To identify the region of AtLORE that mediates its homomerization, we generated truncated variants of AtLORE containing different combinations of ICD, ECD or TMD (Fig. 2A). Full length AtLORE-Km-HA co-immunoprecipitates with full length AtLORE-Km-GFP, AtLORE-ECD-TMD-GFP and weakly with AtLORE-TMD-ICD-Km-GFP upon transient expression in N. benthamiana leaves (Fig. 2B). No co-immunoprecipitation is detected with apoplastic AtLORE-ECD-GFP, cytosolic AtLORE-ICD-Km-GFP or cytosolic GFP. This indicates that ECD and TMD contribute to LORE homomerization. In FRET-FLIM experiments, both AtLORE-ECD-TMD-mCherry and AtLORE-TMD-ICD-Km-mCherry significantly reduce the GFP fluorescence lifetime of the FRET donor AtLORE-Km-GFP (Fig. 2C and S3). Furthermore, AtLORE-ECD-TMD lacking the ICD can still homomerize and interact with AtLORE-TMD-ICD (Fig. 2D). In the latter case, only the TMDs can serve as an interaction interface, yet GFP fluorescence lifetime is reduced, highlighting the contribution of both ECD and TMD to AtLORE homomerization.

A. lyrata and A. halleri have LORE orthologues, but do not respond to 3-OH-C10:0

Phylogenetic analysis shows that LORE is restricted to the plant family of Brassicaceae (Ranf et al., 2015). Putative LORE orthologues are encoded in the genomes of Arabidopsis halleri (AhaLORE, Araha.6790s0007.1), Arabidopsis lyrata (AlyrLORE, AL2G04470) and Capsella rubella (CrubLORE, CARUB_v10021901mg) (Fig. S1). Therefore, we analyzed these species for the production of reactive oxygen species (ROS) (Fig. 3A), which is a typical PTI response to application of 3-OH-C10:0 in A.thaliana (Kutschera et al., 2019). Interestingly, we found that, in contrast to C. rubella and A. thaliana, A. lyrata and A. halleri do not respond to 3-OH-C10:0 elicitation. All four species produced ROS upon flg22 treatment (Fig. S4B). This suggests that functional PTI signaling pathways (Yu et al., 2017) and components for ROS production are in principle functional. That A. lyrata and A. halleri do not respond to 3-OH-C10:0 can originate from diverse failures, such as lack of expression, morphological differences that hinder the access of 3-OH-C10:0 to the PRR or a dysfunctional LORE protein. For further analysis, we extracted RNA from untreated leaf material of C. rubella, A. lyrata and A. halleri and transcribed it into cDNA to clone the coding sequence (CDS) of LORE. We obtained full length CDS clones of all orthologues, confirming that LORE is expressed in A. lyrata and A. halleri leaf tissue used for the ROS assay (Fig. 3A). Amino acid sequences of AlyrLORE and AhaLORE slightly varied from publicly available database sequences (Fig. S8). Furthermore, transient expression of GFP-fusions of
LORE orthologues in *N. benthamiana* show that they localize to the plasma membrane, *AhaLLORE-GFP* was partially mislocalized ([Fig. S4A](#)). Solanaceous *N. benthamiana* has no putative LORE orthologue and is therefore insensitive to 3-OH-C10:0 but can gain the function of 3-OH-C10:0 sensing by transient expression of *AtLORE* ([Ranf et al., 2015](#); [Kutschera et al., 2019](#)). Therefore, we tested the cloned LORE orthologues for functionality in gain-of-function (GOF) ROS assays in *N. benthamiana* ([Fig. 3B](#)). Expression of *AtLORE* or *CrubLORE*, but not *AhaLLORE* or *AlyrLORE* resulted in a ROS response to treatment with 3-OH-C10:0. Taken together, these data support that the LORE orthologues of *A. lyrata* and *A. halleri* are the underlying factor in the inability of these species to respond to 3-OH-C10:0.

**LORE orthologues have elicitor binding capacity**

To identify the region of *AlyrLORE* that causes its dysfunction, we created reciprocal domain swaps (DS) between ICD and ECD of *AtLORE* and *AlyrLORE* and tested those domain swaps (DS) for their GOF-ROS responses in *N. benthamiana* ([Fig. 4A](#)). Interestingly, we could render *AtLORE* inactive by replacing the ECD and TMD with *AlyrLORE*-ECD-TMD. In contrast, *AtLORE* remains functional, when the *AtLORE*-ICD is substituted by *AlyrLORE*-ICD. From this we can conclude that *AlyrLORE* has a signaling-competent kinase domain and a dysfunctional ECD. Next, we tested whether the *AlyrLORE*-ECD is compromised in ligand binding. We have previously shown with ligand-depletion assays that *AtLORE* and *CrubLORE*, but not closely related *AtSD1-23* (AT1G61390) can bind 3-OH-C10:0 ([Shu et al., 2021](#)). Here, we additionally tested the 3-OH-C10:0 binding capacities of the ECDs of *AlyrLORE* and *AhaLLORE* expressed and harvested from *N. benthamiana* apoplasts in ligand-depletion assays. Equal amounts of total protein ([Fig. S5](#)) were incubated with 3-OH-C10:0 and mixtures were filtered through membranes with a 30 kDa molecular weight cut-off. The presence of unbound 3-OH-C10:0 in the filtrate was tested in a bioassay by measuring the cytosolic calcium response of *AtLORE*-overexpressing (OE) *A. thaliana* seedlings. When the ECDs bind the elicitor, the filtrates do not contain 3-OH-C10:0 and do not elicit a calcium response. Interestingly, the ECDs of all tested LORE orthologues bind 3-OH-C10:0 and completely deplete 3-OH-C10:0 from the filtrates, as no calcium responses is detectable after filtrate application ([Fig. 4B](#)). Since neither the kinase activity nor the ligand binding of *AlyrLORE* appears to be affected, the impaired 3-OH-C10:0 perception of *AlyrLORE* must have other causes.

**LORE orthologues from 3-OH-C10:0 nonresponsive species cannot homomerize**

Interestingly, unlike overexpression of *AtLORE* or *CrubLORE*, overexpression of *AlyrLORE* or *AhaLLORE* in *N. benthamiana* does not cause cell death, as determined by chlorophyll fluorescence measurements ([Fig. 5A](#)). This indicates that receptor activation, relevant for the autoimmunity phenotype, is affected in these species. Therefore, we hypothesized that *AlyrLORE* and *AhaLLORE* may fail to homomerize and analyzed their ability to homomerize using FRET-FLIM experiments in *N. benthamiana* ([Fig. 5B-D](#)). Indeed, *AlyrLORE* and *AhaLLORE* are unable to form homomers in *vivo*, whereas signaling-competent *CrubLORE* homomerizes similarly to *AtLORE*. Thus, we conclude that homomerization of LORE is crucial for the activation of signal transduction. *AlyrLORE* and *AhaLLORE* can bind 3-OH-C10:0 but do not homomerize. Thus, ligand binding is independent of receptor homomerization and not sufficient to activate LORE-dependent signaling. Furthermore, our data indicate that the cell death phenotype in *N.*
benthamiana can be used as indicator of LORE receptor functionality and requires both, an active kinase domain and receptor homomerization.

Mapping of homomerization region using AflLORE and AlyrLORE chimera

Our data suggest that loss of the homomerization ability of LORE renders two Arabidopsis species, A. lyrata and A. halleri, insensitive to 3-OH-C10:0. While AyrLORE and AhalLORE share a very high overall amino acid identity with AflLORE, they have several single amino acid polymorphisms (SAPs) in their ECDs and TMD compared to the AflLORE-ECD, distributed across all domains (Fig. S1). To narrow down the region of the ECD that causes the loss of AyrLORE homomerization, we generated chimera between AflLORE and AyrLORE ECDs and TMDs. We either exchanged individual domains, such as lectin 1, lectin 2, EGF or PAN domain (L1, L2, E, P, Fig. S7A) or combinations of domains (LL, EP, Fig. 6A). After verifying the expression and correct localization in N. benthamiana epidermal cells (Fig. S6), we analyzed homomerization of these chimera in FRET-FLIM experiments (Fig. 6 and S7B). Chimera with individual domain swaps all retained their ability to homomerize (Fig. S7B). This indicates that loss of homomerization in AyrLORE is not caused by a single SAP or diversification of a single domain. Therefore, we tested domain combinations, namely LL, EP and full ECD-TMD swaps. Interestingly, substitution of the whole ECD of AflLORE by AyrLORE, but not partial ECD swaps (LL, EP) impair homomerization in FRET-FLIM experiments (Fig. 6B). A similar outcome was observed, when we tested these chimeras in 3-OH-C10:0-triggered ROS or chlorophyll fluorescence assays upon overexpression in N. benthamiana (Fig. 4A, 6C and D). Chimera with partial ECD swaps remain signaling competent, while those with ECD-TMD exchange do not. Hence, we could not pin down a specific region of the AyrLORE-ECD or TMD that causes the loss of homomerization. This might indicate a large, ectodomain-spanning interaction interface of AflLORE.

Homomerization is essential for AflLORE downstream signaling

Collectively, our data suggests that LORE homomerization is essential for its activation and downstream signaling. To confirm this, we performed competition experiments with the truncated AflLORE-ECD-TMD variant. We have shown that AflLORE-ECD-TMD lacking the ICD can form hetero-complexes with full length AflLORE (Fig. 2B and C). The hetero-complexes are presumably signaling incompetent as they contain only one kinase domain. To outcompete AflLORE homocomplexes, AflLORE-ECD-TMD must be present in excess relative to AflLORE. We exploited the fact, that upon transient expression in N. benthamiana AflLORE-ECD-TMD accumulates to higher protein levels than full length AflLORE (Fig. 1B). To enhance the effect, we increased the ratio of Agrobacteria carrying the competitor expression plasmid (AflLORE-ECD-TMD) to those with full length AflLORE to 5:1. Indeed, AflLORE-ECD-TMD outcompetes signaling-competent full length AflLORE homomers in competition assays, as shown by impaired ROS production in N. benthamiana (Fig. 7). This is not observed when cytosolic mCherry is co-expressed in excess. AflLORE-ECD-TMD thus exerts a dominant-negative effect on the ROS response upon elicitation with 3-OH-C10:0. Hence, homomerization of kinase-active LORE is required to activate signaling upon 3-OH-C10:0 elicitation.
**DISCUSSION**

AtLORE is the first SD-RLK from *A. thaliana* with a known ligand and characterized function (Ranf et al., 2015; Kutschera et al., 2019), which makes it an important model for studying signaling mechanisms of the SD-RLK family. However, the mechanism of AtLORE receptor activation remains largely unknown. Here we show that AtLORE forms receptor homomers *in vivo* and that homomerization is required to activate immune signaling. In plants, numerous examples of receptor-coreceptor hetero-dimerization have been found, making it a predominant concept of receptor activation mechanisms (Burkart and Stahl, 2017; Wan et al., 2019; Gou and Li, 2020). In contrast, the receptor activation by homo-dimerization, as shown here for AtLORE, seems to be common among representatives of the SD-RLK family. The SD-RLKs OsLSK1 homomerizes and heteromerizes with five close homologs *in yeast* and *in planta* (Zou et al., 2015). Since its ligand and physiological function are unknown, the mechanistic relevance of the observed interactions also remains unclear. Homo-dimerization of BraSRKs has been studied in detail and was shown to be required for ligand binding and signal transduction (Giranton et al., 2000; Naithani et al., 2007; Shimosato et al., 2007; Ma et al., 2016a; Murase et al., 2020). To date, no co-receptors of any SD-RLK have been identified, which underlines the relevance of homo-dimerization for receptor activation. Protein homomerization analysis is particularly challenging due to uncontrollable homodimer stoichiometry. Most protein-protein interaction methods require two different protein epitope tags, whether for FRET, Co-IP, or BiFC (Xing et al., 2016). However, the combinations of protein-tags in a homomer/dimer is subject to random distribution. Statistically, only one-third of dimers have a combination of two different tags when expressed equimolar. Therefore, large proportions of homomers remain unconsidered or even influence the readout of the experiment. For example, in Co-IPs homodimers with identical tags compete with homodimers containing different tags for binding to the antibody trap, but only the latter are detectable on the immunoblot. In case of FLIM the stoichiometry of homo-dimerization results in formation of FRET-incapable (GFP-GFP/mCherry-mCherry) or FRET-capable (GFP-mCherry) pairs. The method does not provide the resolution to distinguish individual molecules. Rather, the measured average GFP lifetime of each pixel is the result of a random mixture of all possible dimers. This might explain, why the GFP lifetime is only moderately reduced for AtLORE homomers compared to the FRET-positive control, as FRET-incapable AtLORE-GFP homodimers diminish the effect of the FRET-capable AtLORE-GFP–AtLORE-mCherry dimers.

BraSRKs are thought to pre-assemble into homo-dimers in a ligand-independent manner and these preformed complexes allow ligand binding and a rapid activation of the receptor (Giranton et al., 2000; Naithani et al., 2007; Shimosato et al., 2007). Ligand binding might enhance BraSRK dimerization by rearrangement of these pre-assembled complexes (Shimosato et al., 2007). However, whether preformed receptor complexes exist prior to ligand binding under physiological conditions is controversially discussed. AtCERK1 forms homo-dimers independent of its ligand chitin upon overexpression in *A. thaliana* (Liu et al., 2012), whereas under physiological conditions AtCERK1 homo-dimerization was fully dependent on AtLYK5 and chitin binding (Cao et al., 2014; Gong et al., 2020). Ligand-independent self-association of AtFLS2 was also reported (Sun et al., 2012). FLIM analysis of AtFLS2 at the membrane did not show homomerization, leading to the speculation that AtFLS2 might only self-associate upon internalization in a ligand-independent manner (Somssich et al., 2015). Our FRET-FLIM data show that AtLORE homomerization is ligand-independent, at least under the experimental conditions used. We could not detect any significant differences in the homomerization state of AtLORE between 10-20 minutes after application of 3-OH-C10:0. Although receptor complex formation should be a rapid process, this timeframe was suggested for comparable FLIM studies (Somssich et al., 2015). The authors
discussed that a relatively large proportion of complexes need to accumulate to change the average lifetime of one pixel in an FLIM image. To avoid strong overexpression which might lead to artifacts as shown for AtCERK1 (Liu et al., 2012), we used an inducible promotor system for an adjustable and more moderate protein expression. To date we can only speculate how AtLORE homimerization acts under physiological conditions as protein expression driven by the endogenous LORE-promotor is below the detection limit for fluorescence-based assays. At present our data do not allow us to draw conclusions about the oligomerization state of AtLORE. However, for BraSRKs oligomerization into higher order complexes was suggested by cross-linking experiments and velocity sedimentation on sucrose gradients (Giranton et al., 2000), but its physiological relevance remains unclear. Such oligomerization dynamics of receptor complex formation was also reported for other receptor complexes, such as AtFLS2-AtBAK1 (Somssich et al., 2015). The plant may maintain receptor complexes in a stable preformed steady-state to ensure rapid signal transduction that is fine-tuned by multiple regulators in higher-order complexes.

Our data suggest that homomerization of AtLORE is mediated by the ECD and TMD. In Co-IPs, neither the apoplastic ECD nor the cytosolic ICD could interact with full-length AtLORE, suggesting that membrane anchoring is required for dimerization. However, it is also possible that the interaction surface of the membrane-bound full-length AtLORE is not accessible for the soluble truncations. Interestingly, AtLORE-ECD-TMD and AtLORE-TMD-ICD can interact in FRET-FLIM experiments. In this combination, only the TMDs provide a potential interaction surface suggesting that the TMD substantially contributes to AtLORE homomerization. TMD helixes represent a typical interaction interface in the hydrophobic environment of the membrane, which often contain conserved TMD interaction motifs (Herrmann et al., 2009; Langosch and Arkin, 2009; Fink et al., 2012). The relevance of TMDs for receptor complex formation is highly discussed in the animal field (Westerfield and Barrera, 2020). Involvement of TMDs in dimerization has also been shown for several plant RLKs. The TMD of the RLK SUPPRESSOR OF BR1-1 (SOBIR1) possesses a GxxG-motif, which mediates high affinity TMD-TMD association and is crucial for its interaction with the RLK Cf-4 (Bi et al., 2015). The TMD of the RLK Arabidopsis CRINKLY 4 (ACR4) homodimerizes (Stokes and Gururaj Rao, 2008) and is specifically required for the interaction with the RLK CLAVATA1 (CLV1) (Stahl et al., 2013). The relevance of TMDs in SD-RLK signaling is underlined by OsPib-d2, in which a single amino acid polymorphism in the TMD (I441M) determines susceptible or resistant alleles towards rice blast. For BraSRKs it was hypothesized that the TMDs may contribute to the preformed SRK dimer, which is then strengthened by ligand binding (Shimosato et al., 2007; Ma et al., 2016a). Interestingly, high-affinity binding of SCR8 peptides was only observable for membrane-bound SRK8 variants containing the ECD, TMD and parts of the juxta-membrane domain, but not for soluble ECDs (Takayama et al., 2001; Shimosato et al., 2007). An artificially dimerizing form of the SRK8-ECD, achieved by integration of a helix-loop-helix zipper domain, exhibited a high SCR binding affinity, which highlights the importance of membrane anchorage for ligand binding and dimerization (Shimosato et al., 2007). BraSRK homodimerization was furthermore shown to be mediated by the ECD in yeast-two-hybrid studies, while kinase domains do not self-associate (Naithani et al., 2007). Especially the PAN and partially the EGF domains, but not the lectin domains exhibited homo-dimerization capacities in yeast (Naithani et al., 2007). For BraSRK9 and engineered BraSRK8, crystal structures of their ECDs show both ligand- and receptor mediated interaction (Ma et al., 2016a; Murase et al., 2020). In both cases, an BraSRK-ECD dimer co-crystallized with two BraSCR peptide ligands, forming a tetrameric 2:2 receptor-ligand complex. Is it suggested that BraSCRs majorly contribute to complex formation by cross-linkage of the BraSRK monomers. Although studies about BraSRKs provide a detailed insight into homo-dimerization, the comparability between AtLORE and BraSRKs is limited as
AtLORE binds a very small chemical compound instead of a peptide ligand. So far, the binding site of 3-OH-C10:0 in the AtLORE-ECD is unknown. Due to the small size of the ligand, one must assume a relatively small interface between the receptor and the ligand, which may not be sufficient to crosslink two AtLORE monomers or to enhance homo-dimerization in a manner comparable to SRK-SCR complexes. Interestingly, we found that A lyrLORE and A ha LORE cannot homo-dimerize but bind the 3-OH-C10:0 ligand. Thus, 3-OH-C10:0 can bind to LORE monomers. One may speculate that dimerization is stabilized by conformational changes rather than ligand-mediated cross-linking. Taken together, it seems reasonable that AtLORE may form pre-assembled complexes in a ligand-independent manner. However, the underlying molecular mechanism of receptor activation remains unclear.

AtLORE homomerization is essential for receptor activation and downstream signaling. We demonstrate that LORE orthologues of 3-OH-C10:0 insensitive species are impaired in their homomerization capacity, and therefore cannot mediate downstream signaling. Truncated AtLORE containing only ECD and TMD exerts a dominant negative effect on 3-OH-C10:0 induced signaling. We assume that this effect is caused by formation of non-functional heterodimers with full-length AtLORE, which lack downstream signaling capacities. Competition for ligand binding seems unlikely given the high ligand concentrations used in our experiments. Similar competition-based phenomena have been repeatedly described for other RLKs. Overexpression of truncated OsLSK1 lacking the kinase domain resulted in increased plant height and yield in rice (Zou et al., 2015). OsLSK1 was shown to interact with itself and five homologous proteins. Truncated OsLSK1 may interfere with signaling of OsLSK1 and the other interacting SD-RLKs or may compete for ligand binding. Upon overexpression of AtFLS2 truncations lacking the LRR domain a similar effect was observable. The truncations associate with full-length AtFLS2 and exert a dominant negative effect on flg22-induced signaling (Sun et al., 2012). Taken together, these studies show that analysis of a dominant negative effect, which is especially applicable for homomerizing RLKs, provides valuable insight into receptor complexes and their signaling mechanisms.

In vitro phosphorylation assay of microsomal membranes shows that oligomerization of membrane-bound recombinant BraSRKs is essential for auto-phosphorylation (Giranton et al., 2000) and that preformed BraSRK oligomers exhibit a basal constitutive auto-phosphorylation. We assume a similar mode of action for AtLORE. Strong overexpression of AtLORE in N. benthamiana results in cell death. We can show that this phenotype depends on both, an active kinase domain and dimerization ability, as it is completely absent in non-homomerizing LORE-orthologues or kinase mutated AtLORE. Induced cell death is usually known as a highly effective mechanism of hypersensitive defense responses after pathogen recognition (Balint-Kurti, 2019). However, the phenomenon of cell death-like symptoms upon RLKs overexpression has been described repeatedly. Overexpression of AtCERK1 in N. benthamiana resulted in cell death in a kinase-dependent manner (Pietraszewska-Bogiel et al., 2013). It was hypothesized that overproduction of AtCERK1 induces ligand-independent homomerization, leading to auto-activation and dysregulated signal transduction. However, this AtCERK1 cell-death phenotype was not apparent upon stable expression in A. thaliana. Two L-type lectin RLKs (LecRKs), AtLecRK-IX.1 and AtLecRK-IX.2, exhibit cell death upon overexpression in both A. thaliana and N. benthamiana (Wang et al., 2015). The cell death correlated with transgene expression levels and was dependent on kinase activity, but not on extracellular lectin domains. Homomerization was not shown for AtLecRK-IX.1 and AtLecRK-IX.2, yet is also shown for other AtLecRKs (Guo et al., 2018). Taken together, this suggests that homomerizing RLKs are in particular prone to spontaneous auto-activation upon strong overexpression, resulting in cell death. In the case of
LORE, we found that this phenotype can serve as a proxy and simple read-out for signaling active receptor complexes.

Interestingly, we found natural variation in 3-OH-C10:0 responsiveness within a subset of Brassicaceae species. *A. lyrata* and *A. halleri* are closely related to *A. thaliana* and have LORE orthologues with a high degree of sequence identity to AtLORE. While trying to decipher the underlying defects in these two 3-OH-C10:0-insensitive species, we found that their orthologues can bind the 3-OH-C10:0 ligand but exhibit defective dimerization capacities, which render them signaling incompetent. Since we found a number of SAPs across the AlyrLORE-ECD, we investigated chimera of AtLORE and AlyrLORE to identify the causal region or SAP. However, only substitution by the entire AlyrLORE-ECD abolishes homomerization. We hypothesize that several SAPs collectively cause the loss of homomerization. From an evolutionary perspective, random single polymorphisms e.g. in the ligand binding site, seem more likely to lead to a loss-of-function mutation than a series of SAPs accumulating in the ectodomain leading to a loss of homomerization. This has been shown for example for FLS2. Flg22 binding capacities to putative FLS2 orthologues were analyzed in several *A. thaliana* accessions. The data suggested a direct correlation between the FLS2 binding capacities and the responsiveness to flg22 (Bauer et al., 2001). While our findings were rather unexpected from an evolutionary perspective, they may shed light on the evolutionary processes of diversification and neo-functionalization in the family of SD-RLKs which is so far rather poorly understood (Xing et al., 2013). This supports that natural variation provides a powerful molecular toolbox to improve our mechanistic understanding of RLK signaling.

**MATERIALS AND METHODS**

Sequence alignments

Multiple amino acid sequence alignments (MSA) (Fig. S1) were performed with Jalview (Version 2.11.1.4 (Waterhouse et al., 2009)). Amino acid sequences of respective proteins were retrieved from the Uniprot database (Consortium, 2020). For LORE, ECD regions excluding signal peptides (SP) were selected according to domain annotations given on Uniprot and in (Naithani et al., 2007) for LORE (see supplementary table 2). SP predictions of BraSRKs were done by SignalP-5.0 server (Almagro Armenteros et al., 2019). MSA of ECDs without SPs was calculated by the MAFFT (multiple alignment using fast Fourier transformation) algorithm (Katoh et al., 2019) with default settings with the in-built alignment tool of Jalview. Amino acid sequence alignment of AlyrLORE and AhaLORE cloned from cDNA and the respective sequences from genome database entries (Fig. S8) was performed using the in-built alignment tool of the SnapGene software (Insightful Science; available at snapgene.com).

Plant material and cultivation

*N. benthamiana*, *A. thaliana* Col-0, *A. halleri* (N9852, from P. Falter-Braun, Helmholtz Center Munich), *A. lyrata* (MN47, from M. Quint, MLU Halle) and *C. rubella* (N22697, from M. Quint, MLU Halle) were sown on standard potting soil mixed with vermiculite (9:1). All Brassicaceae seeds were stratified in the dark for at least 48 h at 4°C and then grown under short day conditions (8 h light, 16 h darkness, 21°C, and 60% relative humidity). *N. benthamiana* was grown under long day conditions (16 h light, 8 h darkness, 24°C, and 60% humidity). Generation of *A. thaliana* Col-0AEQ overexpressing AtLORE (CaMV35S:LORE, LORE-OE) was previously described (Shu et al.,
For cytosolic Ca\(^{2+}\)-measurements, seeds were surface-sterilized with chlorine gas (4 h) and seedlings grown in liquid medium (0.5× Murashige & Skoog medium, including vitamins (Duchefa), 0.25% sucrose, 1 mM MES, pH 5.7) under long day conditions as described above.

**Molecular cloning**

All plasmids used in this study were cloned using Golden-Gate (GG) (Engler et al., 2009; Weber et al., 2011) or Gateway™ cloning techniques (Katzen, 2007). Cloning of AtLORE (AT1G61380), AtGST (AT1G17170) was cloned from pGEX vector. All plasmids used in this study were cloned using Golden-Gate (GG) (Engler et al., 2009; Weber et al., 2011) or Gateway™ cloning techniques (Katzen, 2007). Cloning of AtLORE (AT1G61380), AtLORE-Km, CrubLORE (CARUB_v10021901mg), CrubLORE-ECD, AtSD1-23i-ECD (AT1G61390) and apoplastic mCherry was described previously (Ranf et al., 2015; Shu et al., 2021). AtGST (AT1G17170) was cloned from pGEX-6P-1. For cloning of AhaLORE (Araha.6790s0007.1) and AylLORE (AL2G04470), total RNA was extracted from leaf material of the respective species by the TRIzol RNA extraction method. RNA samples were treated with DNase I (Thermo Fisher Scientific) and reverse-transcribed into cDNA using RevertAid reverse transcriptase, Ribolock RNase inhibitor and Oligo(dT)\(_{18}\) primer, according to manufacturer’s instructions (Thermo Fisher Scientific). Full length coding sequences (CDS) of respective genes were amplified with specific primers (Supplementary table 1) and ligated into a suitable GG-vector. Sequences cloned from cDNA of AhaLORE and AylLORE slightly varied from database entries (see full sequence alignments figure S8). Conserved ATP binding sites of LORE kinase domains (K516A, Km, primers see supplementary table 1) were modified by site-directed mutagenesis as previously described (Ranf et al., 2015) (primers supplementary table 1). For AhaLORE the first intron of AtLORE (109 bp) was amplified from genomic DNA and inserted into AhaLORE CDSs (at the 45th codon) to circumvent problematic read through in *E. coli* or *Agrobacterium tumefaciens* (primers supplementary table 1). AtLORE truncations and receptor chimera with AylLORE were designed according to domain annotations from Uniprot and Naithani et al. (2007) (see supplementary table 2). For cloning of truncations and chimera respective parts of the CDS were amplified by PCR with BpiI linkers from vectors with full length CDS (backbone PCR) and re-circularized by GG techniques (see Supplementary table 1 and 3). For all membrane bound truncations, the AtLORE signal peptide was integrated. CDSs excluding STOP codons were combined with CaMV35S promoter and terminator, a C-terminal ten-glycine linker and respective C-terminal epitope tags (GGFP, mCherry, SPYCE, SPYNE, hemagglutinin (HA)) using GG techniques. For FRET-FLIM experiments, CDSs were transferred via Gateway™ cloning (LR-clonase™, Thermo Fisher Scientific, according to manufacturer’s manual) into a final Gateway™ vector containing an estradiol inducible trans-activator cassette (XVE) and C-terminal GF, mCherry or GFP-mCherry fusion epitope tags (FLIM-vectors, previously described in Bleckmann et al., 2009)). Final expression vectors were transformed into *Agrobacteria tumefaciens* for transient expression in *plantae*.

**Transient protein expression in N. benthamiana**

*N. benthamiana* was transiently transformed by *Agrobacterium*-mediated transformation as described previously (Shu et al., 2021). *Agrobacterium tumefaciens* (strain GV3101 pMP90) carrying expression constructs were cultivated on LB agar with 30 μg/mL gentamycin, 10 μg/mL rifampicin, and 50 μg/mL kanamycin, or 100 μg/mL spectinomycin for *Agrobacteria* carrying FLIM expression constructs. OD\(_{600}\) of *Agrobacteria* was adjusted to 0.5 for general purposes or 0.025 for gain-of-function ROS measurement. Prior to infiltration, *Agrobacteria* carrying desired expression vectors or the silencing suppressor p19 were mixed in a 1:1 ratio. In case of co-
expression, the ratio was adjusted to 1:1:1. For ROS competition assays, the OD₆₀₀ of Agrobacteria carrying p19 was 0.15, for AtLORE 0.025 and for AtLORE-ECD-TMD or cytosolic mCherry 0.125 (ratio p19:AtLORE:competing component 6:1:5). Expression of FLIM constructs driven by the inducible XVE trans-activator was induced by infiltration of 20 µM β-estradiol and 0.1% Tween20 into transformed leaf areas 24 h after infiltration of agrobacteria. Protein expression was observable 16-24 h after induction.

Protein extraction
Plant material was harvested (60-70 leaf discs of Ø 4 mm), frozen in liquid nitrogen and ground to fine powder (1 min at 30 Hz in a TissueLyzerII, Qiagen). Total protein was extracted by incubation with extraction buffer (6 µL/leaf disc, 150 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet-P40, 10 mM EDTA, 1 mM Na₂MoO₄, 1 mM NaF, 1 mM DTT, 1% (w/v) polyvinylpyrrolidion, 1% (v/v) protease inhibitor cocktail P9599 (Sigma-Aldrich)) for 1 h at 4°C. After centrifugation (18000 g, 30 min, 4°C), the supernatant was used for verification of protein expression via immunoblot or Co-IP experiments.

Co-immunoprecipitation
For Co-IPs, protein extracts were incubated for 1-2 h at 4°C with GFP-Trap_MA (magnetic beads, ChromoTek, handling according to manufacturer’s manual). Magnetic beads were washed three times with buffer (150 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.5% Nonidet-P40) and re-suspended in 20 µL 1xSDS-sample buffer (5x SDS sample buffer: 60 mM Tris-HCL pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenolblue).

Immunoblot
Protein samples in 1x SDS sample buffer were denatured for 10 min at 95 °C and separated by SDS-polyacrylamide gel electrophoresis (5% stacking gel, 10% resolving gel, 60-100 V, 1x Laemmli running buffer). Proteins were blotted onto a 0.2 µm Protran™ nitrocellulose membrane (GE healthcare) using 1x transfer buffer (3.03 g/L Tris base, 14.4 g/L glycine, 20% methanol, 0.05% SDS) and a semi dry transfer cell (Bio-Rad, 1 mA/cm², 1 h). Membrane was blocked with protein free blocking solution T20 (Pierce) and proteins were detected with respective antibodies diluted in blocking solution for primary antibodies or 1x TBS-T (6.06 g/L Tris base, 8.76 g/L NaCl, pH 6.7, 0.05% Tween20) for secondary antibodies (1:1000 anti-GFP 3H9 (ChromoTek) with 1:20000 anti-rat-HRP (A9542, Sigma-Aldrich); 1:500 c-Myc 9E10 (Santa Cruz) with 1:5000 anti-mouse sc-2031 (Santa Cruz); 1:2000 anti-HA-HRP 3F10 (Sigma-Aldrich)). After washing of membranes (3x 10 min in 1x TBS-T), chemiluminescence was detected with a CCD camera system (Fusion SL System, Vilber Lourmat GmbH) upon incubation with a peroxidase substrate (SuperSignal®West Femto Maximum Sensitivity Substrate (Fig. 1A, S2D) or SuperSignal®West Dura Extended Duration Substrate (Pierce) (Fig. 2B)). When immunoblots were analyzed with a second, different antibody, membranes were washed (1x TBS-T) after detection and incubated in stripping buffer (2% SDS, 62.5 mM Tris/HCl pH 6.7, 100mM β-mercaptoethanol) at 50°C for 30 minutes. Stripped membrane was blocked and immunodetected again. Membranes were stained for total protein with amido black (1 g/L amido black, 250 mL/L isopropyl, 100 mL/L acetic acid).
Bimolecular fluorescence complementation

SPYCE/SPYNE epitope tags for BiFCs were previously described (Walter et al., 2004) and adapted for GG cloning. Respective interaction candidates were transiently co-expressed in *N. benthamiana*. YFP fluorescence complementation was visually assessed two days post infiltration via confocal laser scanning microscopy (Leica TCS SP5, ex 514 nm, em 525 – 550 nm, Z-stack of 22 planes). For YFP fluorescence quantification, leaf discs (Ø 4 mm) were floated on water in a black 96-well plate and fluorescence was measured with a plate reader (Tecan Infinite F200 PRO, excitation 485 nm, emission 535 nm, 25 flashes, integration time 20 µs, gain set to 70, 2x2 reads per well). Protein expression was validated by immunoblots.

Trypan blue staining

Leaf discs (Ø 2 cm) were harvested five days post transformation and incubated (95°C, 5 min) with 3 mL trypan blue staining solution (25% (v/v) lactic acid, 25% (v/v) phenol, 25% (v/v) glycerol, 25% (v/v) H₂O, 25% (w/v) trypan blue). Leaves were washed several times with destaining solution (250 g chloral hydrate in 100 mL H₂O) and rinsed with water before photo-documentation.

ROS measurement

Accumulation of ROS was measured with a luminol based reporter system and a microplate reader (Tecan Infinite F200 PRO or Luminoscan Ascent 2.1, Thermo Fisher Scientific) as describe previously (Ranf et al., 2015). Leaf discs (Ø 4 mm) of Brassicaceae (6-8 weeks old) or transiently transformed *N. benthamiana* (36 h post agro-infiltration) were floated on water in white 96-well plates for at least 6h (for *N. benthamiana*) or overnight (for Brassicaceae). Prior to measurements, water was replaced with 100 µL horseradish peroxidase-luminol solution (2 µg/mL horseradish peroxidase, 10 µM L-012 (WAKO Chemicals GmbH)). After addition of elicitors or respective controls (3-OH-C10:0 (Matreya LLC) in MeOH, flg22 (QRLSTGSRINSAKDDAAGLQIA in ddH₂O), luminescence was recorded in relative light units (RLU) in 1 minute intervals for up to 60 minutes. Total ROS accumulation was summed up for elicitor and control treatments.

Ligand depletion assay

Ligand depletion assay was performed as previously described (Shu et al., 2021). ECDs of respective proteins were transiently expressed in *N. benthamiana* apoplasts and harvested via apoplastic washing fluids (AWF). AWFs were concentrated and incubated with 3-OH-C10:0 (9:1, v/v). Unbound elicitor was separated by filtration (30 kDa molecular weight cut-off). Filtrates were used for elicitation of cytosolic calcium signaling in LORE-OE reporter lines as described before (Ranf et al., 2015; Shu et al., 2021). For each time point, luminescence was normalized to total luminescence counts remaining (L/L_{max}) and total accumulation of calcium signals was summed up from 3 to 30 minutes. 5 µL of concentrated AWF with a total protein concentration of 1.5 mg/mL was analyzed for protein expression by immunoblot.
Red light chlorophyll fluorescence measurement for cell death quantification

Red light emission of chlorophyll fluorescence can be measured for quantification of cell death (Landeo Villanueva et al., 2021). Leaf discs (Ø 4 mm) of transiently transformed *N. benthamiana* were floated on water in black 96-well plates and chlorophyll fluorescence was measured with a plate reader (Tecan Infinite F200 PRO, excitation 535 nm, emission 590 nm, 25 flashes, integration time 20 µs, 4x4 reads per well, gain set to 80) as relative fluorescence units (RFU). Values of all reads per well were summed up.

Microscopy

Confocal laser scanning microscopy was performed using either a Leica TCS SP5 (Argon laser, HyD2 detectors) or an Olympus FV3000 system (diode lasers, PMT detectors). Images were acquired with an image size of 512x512 pixels. In case of co-expression analysis, images were taken with a sequential scan to avoid ‘bleed through’. GFP was excited at 488 nm and emission detected at 500-540 nm. mCherry was excited at 561 nm and emission detected at 570-620 nm. YFP was excited at 514 nm and emission detected at 525-550 nm. Images were imported and processed with OMERO (Allan et al., 2012).

Fluorescence lifetime imaging (FLIM)

GFP fluorescence lifetimes were measured via time-correlated single-photon counting (TCSPC) by an Olympus FV3000 system linked to a PicoQuant FCS/FLIM-FRET/rapidFLIM upgrade kit, based on previously described protocols (Weidtkamp-Peters and Stahl, 2017). TCSPC was performed with a 485 nm (LDH-D-C-485) pulsed laser, two TCSPC modules (TimeHarp 260 PICO Dual, TimeHarp 260 NANO Dual) and two photon counting PMA hybrid 40 detectors. Co-expressing cells were detected by confocal imaging via an Olympus FV3000 60x water immersion objective (UPLSAPO60XW 60x/NA 1.2/WD 0.28) and a selected measuring area was magnified (4x zoom). TCSPC was performed with a laser pulse rate of 40.00 MHz, a TCSPC resolution of 25.0 ps and an image size of 512x512 pixels. 500-1000 photon counts per pixel were acquired for each image. FLIM was analyzed by the Symphotime64 software of PicoQuant via n-exponential reconvolution and an internally calculated instrument response function (IRF). A two-parameter fitting (n=2) was performed in most cases. Only FLIM analysis with fitting coefficients ($\chi^2$) between 1.0 and 2.0 were accepted. Intensity weighted average lifetimes $\tau$ of membrane ROIs were determined for each FLIM image.
**FIGURES**

**A** Chlorophyll fluorescence measurements (relative fluorescence units, RFU) in *N. benthamiana* upon transient expression of candidates four days post transformation. Pooled data of two technical replicates from one biological replicate are shown. Data show mean with SD. n=32 leaf discs. Statistics analyzed by one-way ANOVA with Tukey’s multiple comparisons test, α=0.01. Data not sharing the same letter are significantly different.

**B** Anti-GFP and anti-mCherry immunoblot of co-immunoprecipitation (GFP-trap) after transient co-expression of interaction candidates in *N. benthamiana*. Total protein was stained with amido black. Km, kinase mutated (K516A).

**C** FRET-FLIM of AtLORE-Km-GFP and indicated candidates fused to an mCherry epitope tag, transiently co-expressed in *N. benthamiana*. Pooled data of five independent biological replicates. Data show mean with SD. n, number of analyzed cells. Statistics analyzed by one-way ANOVA with Tukey’s multiple comparisons test, α=0.01. Data not sharing the same letter are significantly different.

**D** FRET-FLIM of AtLORE-Km-GFP with AtLORE-mCherry, transiently co-expressed in *N. benthamiana* and treated with 5 µM 3-OH-C10:0 or H₂O as control. FLIM images were acquired 10-20 minutes after treatment. Pooled data from two independent biological replicates. n, number of analyzed cells. Data show mean with SD. Differences between treatments were analyzed by two-way ANOVA with Sidak’s multiple comparisons test, α=0.01.

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**Figure 1** AtLORE forms homomers and has an auto-immunity phenotype in *N. benthamiana*. A Chlorophyll fluorescence measurements (relative fluorescence units, RFU) in *N. benthamiana* upon transient expression of candidates four days post transformation. Pooled data of two technical replicates from one biological replicate are shown. Data show mean with SD. n=32 leaf discs. Statistics analyzed by one-way ANOVA with Tukey’s multiple comparisons test, α=0.01. Data not sharing the same letter are significantly different. B Anti-GFP and anti-mCherry immunoblot of co-immunoprecipitation (GFP-trap) after transient co-expression of interaction candidates in *N. benthamiana*. Total protein was stained with amido black. Km, kinase mutated (K516A). C FRET-FLIM of AtLORE-Km-GFP and indicated candidates fused to an mCherry epitope tag, transiently co-expressed in *N. benthamiana*. Pooled data of five independent biological replicates. Data show mean with SD. n, number of analyzed cells. Statistics analyzed by one-way ANOVA with Tukey’s multiple comparisons test, α=0.01. Data not sharing the same letter are significantly different. D FRET-FLIM of AtLORE-Km-GFP with AtLORE-mCherry, transiently co-expressed in *N. benthamiana* and treated with 5 µM 3-OH-C10:0 or H₂O as control. FLIM images were acquired 10-20 minutes after treatment. Pooled data from two independent biological replicates. n, number of analyzed cells. Data show mean with SD. Differences between treatments were analyzed by two-way ANOVA with Sidak’s multiple comparisons test, α=0.01.
Figure 2 Homomerization of AtLORE is mediated by the extracellular and trans-membrane domain. A Scheme of AtLORE truncation variants; ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain. Created with BioRender.com. B Anti-GFP and anti-mCherry immunoblot of co-immunoprecipitation (GFP-trap) after transient co-expression of full length and truncated AtLORE-Km interaction candidates in N. benthamiana. Total protein is stained with amido black. C, D FRET-FLIM of AtLORE-Km-GFP full length or AtLORE-ECD-TMD-GFP versus truncated AtLORE-mCherry variants transiently expressed in N. benthamiana. Pooled data from two independent biological replicates each. Data show mean with SD. n, number of analyzed cells. Statistics analyzed by one-way ANOVA with Tukey’s multiple comparisons test, α=0.01, data not sharing the same letter are significantly different.
Figure 3 3-OH-C10:0 responsiveness of *A. lyrata*, *A. halleri* and *C. rubella* or *N. benthamiana* expressing putative LORE orthologues. A Total ROS accumulation of *A. halleri*, *C. rubella*, *A. lyrata* and *A. thaliana* leaf discs treated with 5 µM 3-OH-C10:0 or MeOH as control. Median with minimum to maximum of total ROS between 3-60 minutes after elicitation is shown; n=8 leaf discs. Significance between MeOH and 3-OH-C10:0 treatment tested with two-way ANOVA with Sidak’s multiple comparisons test, α=0.01. B Total ROS accumulation of *N. benthamiana* leaf discs transiently overexpressing *AtLORE* or putative LORE orthologues upon application of 1 µM 3-OHC10:0 or MeOH as control. Boxplot displays minimum to maximum with median of total ROS between 3-45 minutes after treatment. n, number of leaf discs. Combined data of two independent datasets from one biological replicate are shown. For *AtLORE* and *AtLORE-Km* data of both datasets were pooled. Statistics was analyzed by two-way ANOVA with Sidak’s multiple comparisons test, α= 0.01.
Figure 4 The ECD of *AlyrLORE* is causal for defective 3-OH-C10:0 perception, but ligand binding of all orthologues is functional. A Total ROS accumulation of *AtLORE*, *AlyrLORE* and chimera with either ECD-TMD or ICD domain swap (DS) elicited with 10 µM 3-OH-C10:0 or MeOH. Median with minimum to maximum is shown; n=12 leaf discs. Statistics was analyzed by two-way ANOVA with Sidak’s multiple comparisons test, α= 0.01. B 3-OH-C10:0 binding to LORE-ECDs was tested in a ligand depletion assay. Unbound 3-OH-C10:0 in filtrates was detected by cytosolic Ca$^{2+}$ measurements of LORE-OE lines. Ligand binding is indicated by full depletion of a Ca$^{2+}$ response. Data show mean and SD of total [Ca$^{2+}$]$_{cyt}$ from 3 to 30 minutes; n=3 seedlings.
Figure 5 Putative LORE orthologues from 3-OH-C10:0 unresponsive species are impaired in homomerization. A Chlorophyll fluorescence measurement of *N. benthamiana* leaves transiently overexpressing AtLORE, AlyrLORE, AhaLORE and CrubLORE. Mean and SD of pooled data from two technical replicates are shown. *n*=32 leave discs. Statistics analyzed by one-way ANOVA with Tukey’s multiple comparisons test, *α*=0.01. Data not sharing data are significantly different. Experiment was repeated two times with similar outcome. B-D FRET-FLIM of putative LORE orthologous from *A. lyrata* (B) *A. halleri* (C) and *C. rubella* (D) transiently expressed in *N. benthamiana*. Km, kinase mutated; i, integrated LORE intron; *n*, number of analyzed cells, *r*, number of biological replicates. Data show mean with SD. Statistics analyzed by one-way ANOVA with Tukey’s multiple comparisons test, *α*=0.01. Data not sharing the same letters are significantly different.
Figure 6 Mapping of dimerization region using receptor chimera of AtLORE and AlyrLORE.

A Schematic overview of chimera between AlyrLORE and AtLORE. DS, domain swap; LL, lectin domain 1 and lectin domain 2; EP, EGF and PAN domain. Created with BioRender.com. B FRET-FLIM of AtLORE-AlyrLORE domain swaps (DSAlyr) transiently expressed in N. benthamiana. Mean and SD of pooled data are shown; r, number of biological replicates; n, number of analyzed cells. Statistics analyzed by one-Way ANOVA with Tukey’s multiple comparisons test, α=0.01. Data not sharing the same letter are significantly different. C Gain of function ROS measurements of AtLORE-AlyrLORE chimera transiently expressed in N. benthamiana upon elicitation with 10 µM 3-OH-C10:0 or MeOH as control. Median with minimum to maximum is shown; n=12 leaf discs. Statistics was analyzed by two-way ANOVA with Sidak’s multiple comparisons test, α= 0.01. D Chlorophyll fluorescence measurements (in relative fluorescence units, RFU) in N. benthamiana upon transient expression of AtLORE-AlyrLORE chimera four days post transformation. Mean and SD of pooled data from two technical replicates of one biological replicate are shown. n, number of leaf discs. Statistics analyzed by one-way ANOVA with Tukey’s multiple comparisons test, α=0.01. Data not sharing the same letter are significantly different.
Figure 7 AtLORE homomerization is essential for downstream signaling. Dominant negative effects of co-expressed AtLORE-ECD-TMD-HA or cytosolic mCherry was analyzed by competitive ROS accumulation measurement in N. benthamiana transiently co-expressing indicated candidates upon elicitation with 5 µM 3-OH-C10:0 or MeOH as a control. Median with minimum to maximum of total ROS between 3 and 45 min from three technical replicates is shown; n=12 leaf discs. Differences between treatments was analyzed by two-way ANOVA and Sidak’s multiple comparisons test (α=0.01).
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REFERENCES


