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A lectin S-domain receptor kinase mediates lipopolysaccharide sensing in *Arabidopsis thaliana*

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The sensing of microbe-associated molecular patterns (MAMPs) triggers innate immunity in animals and plants. Lipopolysaccharide (LPS) from Gram-negative bacteria is a potent MAMP for mammals, with the lipid A moiety activating proinflammatory responses via Toll-like receptor 4 (TLR4). Here we found that the plant *Arabidopsis thaliana* specifically sensed LPS of *Pseudomonas* and *Xanthomonas*. We isolated LPS-insensitive mutants defective in the bulb-type lectin S-domain-1 receptor–like kinase LORE (SD1-29), which were hypersusceptible to infection with *Pseudomonas syringae*. Targeted chemical degradation of LPS from *Pseudomonas* species suggested that LORE detected mainly the lipid A moiety of LPS. LORE conferred sensitivity to LPS onto tobacco after transient expression, which demonstrated a key function in LPS sensing and indicated the possibility of engineering resistance to bacteria in crop species.

Yield losses due to infestation with pathogenic microorganisms are of major agro-economical concern. Plant breeding aims to produce resistant varieties by exploiting the plant's natural defense repertoire to diminish the need for the application of chemical pesticides. Lacking an adaptive immune system, plants rely mainly on innate immunity[1,2]. They have evolved diverse immunoreceptors to detect microbial invaders via microbe-associated molecular patterns (MAMPs), microbial effectors and microbe-induced cellular perturbations. MAMPs, generally common to a wide range of pathogens and conserved due to their essential functions for the pathogen, are detected by specific pattern-recognition receptors (PRRs) at the cell surface and induce pattern-triggered immunity (PTI). To infect host plants, pathogens have evolved effectors to subvert PTI.

Genome analysis has revealed a disproportionate expansion in the number of genes encoding cell-surface receptors during the evolution of land plants, with >400 receptor-like kinases (RLKs) and >170 receptor-like proteins (RLPs) in *Arabidopsis thaliana*[3,4]. RLKs are membrane-spanning proteins with a cytoplasmic kinase domain for intracellular signaling and diverse extracellular domains with ligand-binding potential. So far, the biological function of only a few RLK-ligand pairs has been elucidated, several of which are involved in PTI. The leucine-rich repeat–containing RLKs (LRR-RLKs) FLS2 and EFR sense the bacterial MAMPs flagellin (or the flagellin-derived peptide flg22) and elongation factor Tu (or the elongation factor Tu–derived peptide elf18), respectively. Both receptors associate with the LRR-RLK BAK1 as coreceptor[5]. Similarly, the lysin motif–containing RLK (LysM-RLK) CERK1 (LYK1) acts in concert with other LysM-RLKs (such as LYK4 and LYK5) or LysM-RLPs (such as LYM1 and LYM3) to sense fungal chitin oligomers or bacterial peptidoglycan, respectively[5–7]. Generally, different MAMPs activate a common set of signaling and defense responses. Rapid changes in the cytosolic concentration of the secondary messenger Ca^{2+} ($[Ca^{2+}]_{cyt}$) are crucial for downstream responses. The subsequent generation of reactive oxygen species (ROS) by the NADPH oxidase RBOHD directly confines pathogen spreading via toxic effects and contributes to signaling. Deposition of the β-1,3-glucan callose leads to fortification of the cell wall. The activation of calcium-dependent kinases and mitogen-activated protein kinases (MAPKs) switches metabolic processes and gene expression to a defensive state[2].

In mammals, several LRR-containing Toll-like receptors (TLRs) sense MAMPs such as flagellin or lipopolysaccharide (LPS; also called 'endotoxin'). LPS, which is abundant in the outer cell envelope of Gram-negative bacteria, consists of lipid A and a core oligosaccharide region that is mostly decorated with an O-polysaccharide (OPS) consisting of many oligosaccharide repeats[8]. The variable OPS exhibits specific antigenic activities in the adaptive immune system[8,9]. The outer core oligosaccharide of *Pseudomonas aeruginosa* LPS is recognized by the chloride- and bicarbonate-ion-channel regulator CFTR[10,11]. The lipid A moiety is sensed as a MAMP by the innate immune system via TLR4 and its coreceptor MD2 and activates proinflammatory responses[12]. An exaggerated, uncontrolled immune reaction to LPS can result in sepsis and life-threatening septic shock[12]. Additionally, certain mammalian caspases have been identified as TLR4-independent intracellular sensors of LPS[13].

LPS also acts as a MAMP in various plant species[14,15]. In the model plant *A. thaliana*, for example, the production of nitric oxide

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is stimulated by LPS from various bacteria as well as by enterobacterial lipid A[16], while the expression of genes encoding defense-related proteins is induced by core oligosaccharides and lipid A from *Xanthomonas campestris* and lipid A from *Burkholderia cepacia*, as well as by OPS rhamnans, found in many phytopathogenic bacteria[16–19]. Thus, plants are able to sense all three parts of LPS. However, the underlying mechanisms plants use to sense these LPS components are yet unknown[14].

Here we found that *A. thaliana* sensitively detected LPS of *Pseudomonas* species and *X. campestris* via the bulb-type (B-type) lectin S-domain (SD)-1 RLK LORE ('lipooligosaccharide-specific reduced elicitation'; The Arabidopsis Information Resource (TAIR) accession code AT1G61380; also known as SD1-29). *A. thaliana* expressing mutant LORE proteins were impaired in LPS-triggered PTI in response to infection with *Pseudomonas syringae*. The phylogeny of LORE-like SD-RLKs suggested that LORE-mediated sensing of LPS is restricted to the plant family Brassicaceae. Transient expression of LORE conferred sensitivity to LPS onto solanaceous tobacco, which indicated that LORE acts as an immunoreceptor of LPS. Finally, we found that the lipid A moiety was required and sufficient for LORE-dependent sensing of LPS.

RESULTS

Pseudomonas LPS acts as MAMP in *A. thaliana*

Studies of *A. thaliana* plants of ecotype Columbia-0 (Col-0) carrying the calcium reporter aequorin (Col-0^{AEQ}) have shown that MAMPs rapidly induce characteristic elevations in $[Ca^{2+}]_{cyt}$ (ref. 20). LPS extracted from several *Pseudomonas* species induced elevations in $[Ca^{2+}]_{cyt}$ in *A. thaliana* seedlings (**Fig. 1a**). The sources of LPS included the plant pathogen *P. syringae*, and *P. fluorescens* and *P. alcaligenes*, which are common bacteria in soil and water, as well as the human pathogen *P. aeruginosa* (**Table 1**). LPS-induced elevations in $[Ca^{2+}]_{cyt}$ were not altered in *A. thaliana* seedlings carrying loss-of-function mutations in genes encoding any of several RLKs (*cerk1-2*, *lyk4*, *fls2-26*, *efr-1* or *bak1-4*) or when LPS preparations had been treated with proteinase K prior to elicitation (**Supplementary Fig. 1a**,**b**). The Ca^{2+}-channel blocker $LaCl_3$ and the kinase inhibitor K-252a inhibited the LPS-induced elevations in $[Ca^{2+}]_{cyt}$ (**Supplementary Fig. 1c**). To identify the structural regions of LPS crucial for sensing by *A. thaliana*, we used *P. aeruginosa* mutants defective in the biosynthesis of LPS core oligosaccharides. The PAN1 mutant strain of *P. aeruginosa*, a rough-type strain, lacks OPS[21]. The phosphoglucomutase Δ*algC* mutant or phage-resistant strains H4 and R5 further lack most of the outer core region[22] (**Supplementary Fig. 1d**). LPS from these mutants triggered elevations in $[Ca^{2+}]_{cyt}$ similar to those elicited by smooth-type LPS containing OPS, such as LPS from *P. aeruginosa* strain PAO1 or Fisher type 1 (**Fig. 1a**). LPS concentrations below 0.5 μg/ml induced $[Ca^{2+}]_{cyt}$ elevations in *A. thaliana* (**Fig. 1b**); these LPS concentrations were much lower than LPS elicitor concentrations reported for plants[14,15]. The size heterogeneity of the repetitive OPS in smooth-type LPS precludes estimation of molar concentrations. However, for rough-type LPS such as that from *P. aeruginosa* strain H4 (main-component molecular size of ~2,800 Da)[23], a concentration of 0.5 μg/ml can be estimated to correspond to 150–200 nM, a concentration typically used for other MAMPs in plants. Thus, *A. thaliana* sensitively detected the conserved inner core–lipid A moiety of *Pseudomonas* LPS[8,22]. In addition to eliciting elevations in $[Ca^{2+}]_{cyt}$, *Pseudomonas* LPS triggered the accumulation of ROS via the NADPH oxidase RBOHD and activation of the MAPKs MPK3 and MPK6, as well as deposition of the plant polysaccharide callose (**Supplementary Fig. 2**). Therefore, *Pseudomonas* LPS initiated responses to MAMPs typically mediated by PRRs in plants[2,20].

Sensing of *Pseudomonas* LPS requires the RLK LORE

Screening for mutants with altered MAMP-induced elevations in $[Ca^{2+}]_{cyt}$ proved suitable for identifying components of PRR complexes[24]. In a genetic screen for mutants with impaired *Pseudomonas* LPS-induced elevations in $[Ca^{2+}]_{cyt}$, we isolated four allelic *A. thaliana* mutants (*lore-1*, *lore-2*, *lore-3* and *lore-4*) that were insensitive to *Pseudomonas* LPS (**Fig. 2a–c** and **Supplementary Fig. 3a**). Genetic mapping identified the product of *LORE* as SD1-29 (TAIR accession code AT1G61380), which belongs to the class of B-type lectin RLKs also known as 'SD-RLKs' (**Supplementary Fig. 3b**). *LORE* is substantially upregulated by treatment with MAMPs and pathogen infection and is coexpressed with the PRR-encoding genes *FLS2* and *EFR*, which supports the proposal of a potential role for LORE in PTI[25]. LORE belongs to the SD1 subfamily of RLKs (SD1-RLKs), which consists of 32 members in *A. thaliana* that share a similar domain structure[4]. The presence of a signal peptide together with a transmembrane domain would suggest localization of SD1-RLKs to the plasma membrane. The extracellular putative ligand-binding region of SD1-RLKs consists of an amino-terminal B-type lectin domain, followed by an

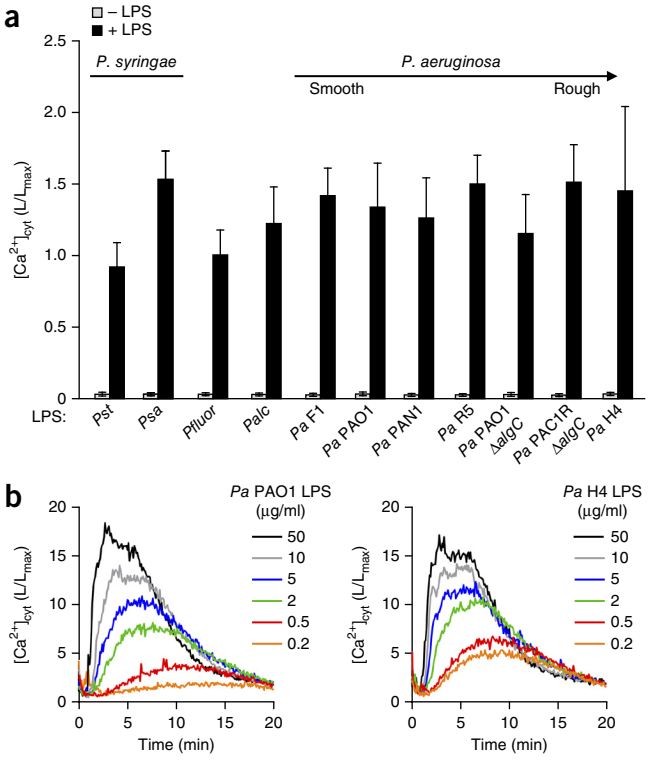

Figure 1 Elevations in $[Ca^{2+}]_{cyt}$ in *A. thaliana* seedlings treated with *Pseudomonas* LPS. (**a**) $[Ca^{2+}]_{cyt}$ in *A. thaliana* Col-0^{AEQ} seedlings 1 min before elicitation with LPS (– LPS) and maximum elevation after application of LPS (15 μg/ml) from various *Pseudomonas* species (**Table 1**) (+ LPS), with reconstitution of aequorin with 5 μM coelenterazine-h; results are presented as luminescence counts per second relative to total luminescence counts remaining (L/L_{max}). (**b**) $[Ca^{2+}]_{cyt}$ over time in Col-0^{AEQ} seedlings treated with various concentrations of LPS (0.2–50 μg/ml) from smooth-type (PAO1) or rough-type (H4) *P. aeruginosa*, with reconstitution of aequorin with 20 μM native coelenterazine. Data are representative of two experiments with similar results (mean and s.d. of $n = 6, 10, 8, 8, 12, 8, 8, 8, 8, 8$ or 8 (left to right) plants per LPS sample in **a**, or mean of $n = 4$ plants per concentration in **b**).

Table 1 Bacterial strains and abbreviations

Bacterial strain	Abbreviation
Pseudomonas aeruginosa Fisher type 1 (Habs 6)	*Pa* F1
Pseudomonas aeruginosa PAO1	*Pa* PAO1
Pseudomonas aeruginosa PAO1 Δ*algC*	*Pa* PAO1 Δ*algC*
Pseudomonas aeruginosa H4	*Pa* H4
Pseudomonas aeruginosa R5	*Pa* R5
Pseudomonas aeruginosa PAN1	*Pa* PAN1
Pseudomonas aeruginosa PAC 1R Δ*algC*	*Pa* PAC1R Δ*algC*
Pseudomonas syringae pv. *tomato* DC3000	*Pst* DC3000
Pseudomonas syringae pv. *apii*	*Psa*
Pseudomonas alcaligenes 537	*Palc*
Pseudomonas fluorescens ATCC49271	*Pfluor*
Xanthomonas campestris pv. *malvacearum* GSPB1386	*Xcm* (1)
Xanthomonas campestris pv. *malvacearum* GSPB2388	*Xcm* (2)
Xanthomonas campestris pv. *phaseoli* var. fuscans GSPB271	*Xcph*
Xanthomonas campestris pv. *begoniae* GSPB525	*Xcb*
Burkholderia cepacia (GIFU645/ATCC25416)	*Bcp*
Burkholderia pseudomallei (GIFU12046/3P-62)	*Bpm*
Escherichia coli K12	*Ec* K12
Escherichia coli O111:B4	*Ec* O111
Escherichia coli KPM 53	*Ec* Kdo_2-LA
Salmonella enterica sv. Typhimurium	*Sm* Typh
Salmonella enterica sv. Minnesota R595	*Sm* R595

S-locus glycoprotein domain or epidermal growth factor-like domain and a plasminogen-apple-nematode domain. The cytoplasmic serine-threonine kinase domain of SD1-RLKs is a so-called 'RD kinase'; i.e., an arginine precedes the conserved aspartate proton acceptor in the kinase domain[2]. A domain of unknown function, DUF3403, constitutes the furthest carboxy-terminal end of SD1-RLKs. The mutations in the *A. thaliana* mutants *lore-2* and *lore-3* led to substitutions of amino acids in the plasminogen-apple-nematode domain and kinase domain of LORE, whereas the LORE expressed by *A. thaliana* mutants *lore-1* and *lore-4* was truncated through premature stop codons after the epidermal growth factor domain or within the kinase domain, respectively (**Supplementary Fig. 3c**). At the functional level, all four *A. thaliana* mutants showed substantially diminished LPS-triggered elevations in $[Ca^{2+}]_{cyt}$ compared with those of the Col-0^{AEQ} plants (**Fig. 2a–c** and **Supplementary Fig. 3a**). In addition, we analyzed the *A. thaliana* mutant line *sd1-29* (The European Arabidopsis Stock Centre (NASC) accession code SAIL_857_E06) in which *SD1-29* is disrupted by an inserted transfer DNA (T-DNA) and that lacks detectable full-length transcripts of *SD1-29* (**Supplementary Fig. 3b,d**). Aequorin-expressing *sd1-29*^{AEQ} plants, generated by crossing of *sd1-29* plants with Col-0^{AEQ} plants, also showed substantially diminished LPS-triggered elevations in $[Ca^{2+}]_{cyt}$ (**Fig. 2b** and **Supplementary Fig. 3e**). A survey of 11 different smooth- and rough-type *Pseudomonas* LPS confirmed diminished elevations in $[Ca^{2+}]_{cyt}$ in *A. thaliana* mutants *lore-1*, *lore-2* and *sd1-29*^{AEQ}, but all mutants responded normally to chitin, flg22 and elf18 (**Fig. 2b**). Whereas measurements of $[Ca^{2+}]_{cyt}$ made with aequorin reconstituted with its 'native' aequorin luminophore coelenterazine showed little elevation in $[Ca^{2+}]_{cyt}$ above the resting concentration in *A. thaliana* mutants *lore-1* through *lore-4* (**Fig. 2a** and **Supplementary Fig. 3a**), use of the more sensitive derivative 'coelenterazine-h' revealed some marginal but variable fluctuations in $[Ca^{2+}]_{cyt}$ in the mutant lines in response to individual LPS preparations (**Fig. 2b** and **Supplementary Fig. 3e**). Additionally, the introduction of a genomic DNA fragment covering the *SD1-29* open reading frame and a 1-kilobase cis-regulatory region upstream of the *SD1-29* open reading frame into the *A. thaliana lore-1*

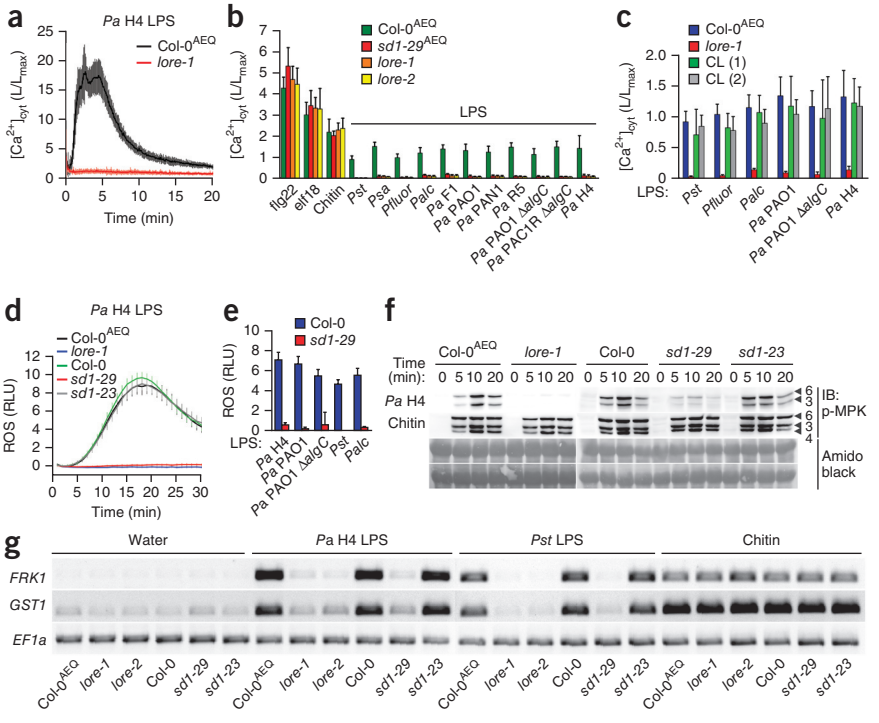

Figure 2 LPS-triggered PTI responses in *A. thaliana*. (**a**) $[Ca^{2+}]_{cyt}$ in Col-0^{AEQ} (control) and *lore-1* mutant *A. thaliana* seedlings treated with LPS from *P. aeruginosa* H4 (50 μg/ml), with reconstitution of aequorin with 20 μM native coelenterazine. (**b**) Maximum $[Ca^{2+}]_{cyt}$ in Col-0^{AEQ}, *sd1-29*^{AEQ}, *lore-1* and *lore-2* mutant *A. thaliana* seedlings after treatment with flg22 (1 μM), elf18 (1 μM), chitin (100 μg/ml) or various LPS preparations (15 μg/ml), with reconstitution of aequorin with 5 μM coelenterazine-h. (**c**) Maximum $[Ca^{2+}]_{cyt}$ in Col-0^{AEQ}, *lore-1* or complementation lines 1 and 2 (CL (1) and CL (2)) after treatment with various LPS preparations (15 μg/ml), with reconstitution of aequorin with 5 μM coelenterazine-h. (**d**) ROS accumulation in leaf discs of control and mutant plants treated with LPS from *P. aeruginosa* H4 (10 μg/ml); results were normalized to ROS before LPS treatment and were calculated by subtraction of ROS of untreated control samples and are presented as relative light units (RLU). (**e**) Maximum accumulation of ROS in leaf discs of Col-0 and *sd1-29 A. thaliana* plants treated with LPS (15 μg/ml) from various *Pseudomonas* species (ROS values calculated as in **d**). (**f**) Immunoblot analysis (IB) of phosphorylated MPK6 (6), MPK3 (3) and MPK4 (4) (arrowheads, right margin; p-MPK) in Col-0^{AEQ}, *lore-1*, Col-0, *sd1-29* and *sd1-23 A. thaliana* seedlings at various times (above lanes) after treatment with LPS from *P. aeruginosa* H4 (20 μg/ml) or with chitin (50 μg/ml); below, amido black staining of total proteins (loading control). (**g**) Expression of the PTI response genes *FRK1* and *GST1* and the control gene *EF1a* in *A. thaliana* seedlings (mutant or control as in **f**) 4 h after treatment with water, LPS from *P. aeruginosa* H4 or *P. syringae* pv. *tomato* (10 μg/ml) or chitin (50 μg/ml). Data are representative of two experiments with similar results (mean and s.d. of $n = 7$ plants per genotype in **a**; $n = 6, 5, 6, 6; 6, 5, 6, 6; 10, 7, 10, 10; 6, 4, 6, 6; 10, 2, 10, 4; 8, 7, 8, 8; 8, 8, 8, 8; 12, 11, 12, 12; 8, 5, 8, 8; 8, 4, 8, 8; 8, 5, 8, 8; 8, 8, 8, 8; 8, 6, 8, 8; 8, 5, 8, 8$ (left to right) plants per genotype and treatment in **b**; $n = 6, 6, 7, 8; 14, 14, 14, 14; 10, 10, 10, 10; 8, 8, 8, 8; 14, 14, 14, 14; 12, 12, 12, 12$ (left to right) plants per genotype and treatment in **c**; mean and s.e.m. $n = 16$ (**d**) or $n = 8$ (**e**) discs from eight plants per genotype).

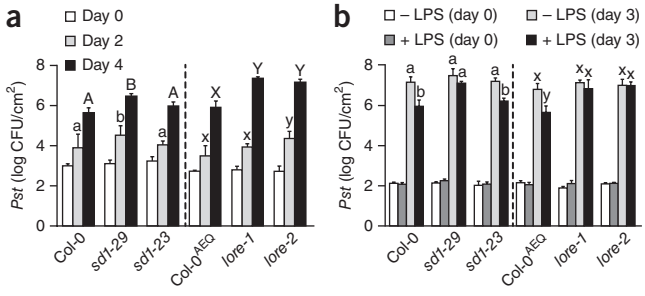

Figure 3 Infection of *A. thaliana* with *P. syringae*. (**a**) Bacterial growth in Col-0, *sd1-29*, *sd1-23*, Col-0^{AEQ}, *lore-1* and *lore-2 A. thaliana* plants on days 0, 2 and 4 after spray inoculation with *Pst* DC3000 (1×10^8 colony-forming units (CFU) per ml inoculum). $P > 0.05$ (not significant) (same letters) or $P < 0.05$ (different letters), with separate analyses for day 2 (lower case) and day 4 (upper case), and for non-reporter lines (a,b,A,B) and aequorin-expressing plant lines (x,y,X,Y) (two-way analysis of variance (ANOVA) (genotype × treatment) and Bonferroni's multiple-comparison post-test). (**b**) Bacterial growth in *A. thaliana* (genotypes as in **a**) treated with LPS from *P. aeruginosa* H4 (25 µg/ml) 1 d before infiltration of *Pst* DC3000 (1×10^5 CFU/ml inoculum), assessed on days 0 and 3. $P < 0.001$ (as in **a**), with separate analyses for non-reporter and aequorin-expressing lines (one-way ANOVA and Tukey's multiple-comparison post-test). Data are representative of four (**a**) or two (**b**) experiments with similar results (mean and s.d. of $n = 4$ for Col-0 (day 2) or $n = 6$ (all others) samples (**a**) or $n = 3$ (day 0) or $n = 6$ (day 3) samples (**b**) randomly obtained from three individual plants per genotype and treatment).

mutant (to generate two complementation lines) restored the $[Ca^{2+}]_{cyt}$ response to *Pseudomonas* LPS (**Fig. 2c**). In line with the substantially diminished elevations in $[Ca^{2+}]_{cyt}$, the LPS-triggered accumulation of ROS, activation of MPK3 and MPK6 and expression of PTI response genes such as *FRK1* and *GST1* were also largely abolished in *A. thaliana* mutants *lore-1*, *lore-2* and *sd1-29* (**Fig. 2d–g** and **Supplementary Fig. 3f**). The paralog most similar to *LORE*, *SD1-23* (TAIR accession code AT1G61390) lies in tandem with *SD1-29*. SD1-23 shares overall 77% amino acid identity with SD1-29, and the potential ligand-binding ectodomain is even 81% identical. However, SD1-23 seemed to not be required for the sensing of LPS, as the T-DNA–insertion mutant *sd1-23* (NASC accession code GK-079G10), which lacked detectable full-length *SD1-23* transcripts (**Supplementary Fig. 3b,d**), responded to LPS with PTI responses comparable to that of the Col-0 control (**Fig. 2d,f,g** and **Supplementary Fig. 3f**). In conclusion, LORE was required for sensing of the conserved inner core–lipid A moiety of *Pseudomonas* LPS.

Sensing of LPS modulates immunity to *Pseudomonas* infection

In line with the diminished responses to MAMPs noted upon treatment with *Pseudomonas* LPS, *A. thaliana* mutants *lore-1*, *lore-2* and *sd1-29* were more susceptible to the virulent *P. syringae* pathogenic variant (pv.) *tomato* DC3000 (*Pst* DC3000) than were their respective Col-0 or Col-0^{AEQ} control, *sd1-23* mutants or the complementation lines described above (**Fig. 3a** and **Supplementary Fig. 4a**). The application of MAMPs induces PTI that results in resistance of *A. thaliana* to pathogens[2,26]. Accordingly, Col-0 and Col-0^{AEQ} control plants pre-treated with *Pseudomonas* LPS were more resistant to subsequent infection with *Pst* DC3000, but this LPS-induced resistance was lost in *A. thaliana* mutants *lore-1*, *lore-2* and *sd1-29* (**Fig. 3b** and **Supplementary Fig. 4b,c**). These results highlighted a role for LORE in LPS-triggered immunity in plants.

Sensing of *Xanthomonas* LPS requires LORE

LPS from *Xanthomonas* species and *Burkholderia* species has been shown to induce defense responses in *A. thaliana*[14]. To further determine the specificity of LORE-mediated sensing of LPS, we assessed LPS preparations from these bacteria as well as enterobacterial LPS for LORE-dependent early PTI responses. *X. campestris* LPS triggered elevations in $[Ca^{2+}]_{cyt}$ and production of ROS in Col-0^{AEQ} and Col-0 control plants (**Fig. 4a,c**). In contrast, no obvious elevation in $[Ca^{2+}]_{cyt}$ or production of ROS was detectable after application of LPS from *B. cepacia* or *Burkholderia pseudomallei* or after application of LPS from enterobacteria such as *Escherichia coli* or *Salmonella enterica*

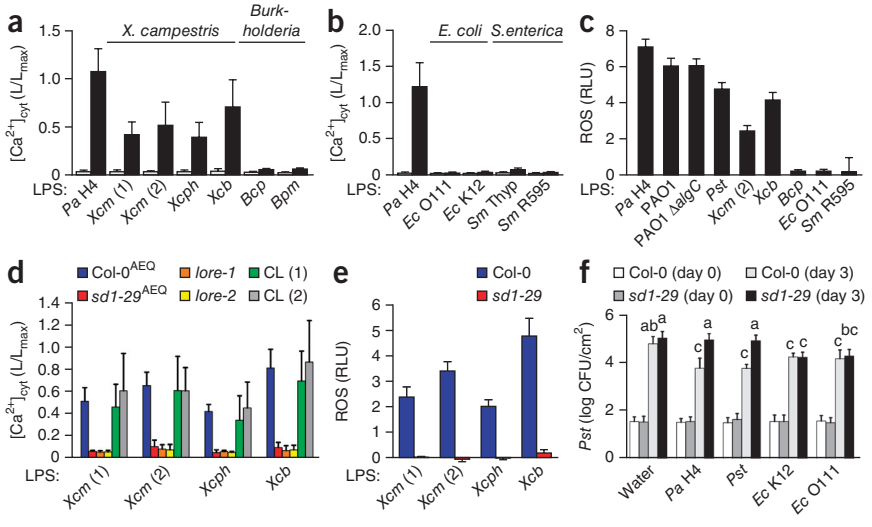

Figure 4 *A. thaliana* PTI responses to LPS from various bacteria. (**a**,**b**) $[Ca^{2+}]_{cyt}$ in Col-0^{AEQ} seedlings treated with LPS from various bacteria (**Table 1**), at a concentration of 25 µg/ml (**a**) or 50 µg/ml (**b**), with reconstitution of aequorin with 5 µM coelenterazine-h. (**c**) Maximum accumulation of ROS in Col-0 leaf discs after treatment with LPS (25 µg/ml) from various bacteria. (**d**) Maximum $[Ca^{2+}]_{cyt}$ in Col-0^{AEQ}, *sd1-29*^{AEQ}, *lore-1* and *lore-2 A. thaliana* seedlings and complementation lines after treatment with LPS (25 µg/ml) from *X. campestris*, with reconstitution of aequorin with 5 µM coelenterazine-h. (**e**) Maximum accumulation of ROS in leaf discs of Col-0 and *sd1-29 A. thaliana* plants induced by LPS (25 µg/ml) from various strains of *X. campestris*. (**f**) Bacterial growth in Col-0 and *sd1-29 A. thaliana* plants treated with water or LPS (25 µg/ml) 1 d before infiltration of *Pst* DC3000 (1×10^5 CFU/ml), measured after 0 or 3 d (two biological replicates of these experiments are in **Supplementary Fig. 4b,c**). $P > 0.05$ (not significant), same letters; $P < 0.05$, different letters (one-way ANOVA and Tukey's multiple-comparison post-test). Data are representative of two experiments with similar results (**a–e**; mean and s.d. of $n = 20, 12, 9, 12, 12, 3, 3$ (**a**) or $n = 18, 12, 12, 6, 12$ (**b**) (left to right) plants per LPS sample; mean and s.e.m. of $n = 16$ discs from eight plants per genotype (**c**); mean and s.d. of $n = 14, 7, 14, 8, 14, 14$; 12, 9, 12, 12, 12, 12; 8, 7, 8, 8, 8, 8; or 18, 9, 17, 12, 18, 18 (left to right) plants per genotype and treatment (**d**); mean and s.e.m. of $n = 8$ discs from eight plants per genotype (**e**)) or one experiment (**f**; mean and s.d. of $n = 6$ samples randomly obtained from three plants per genotype and treatment).

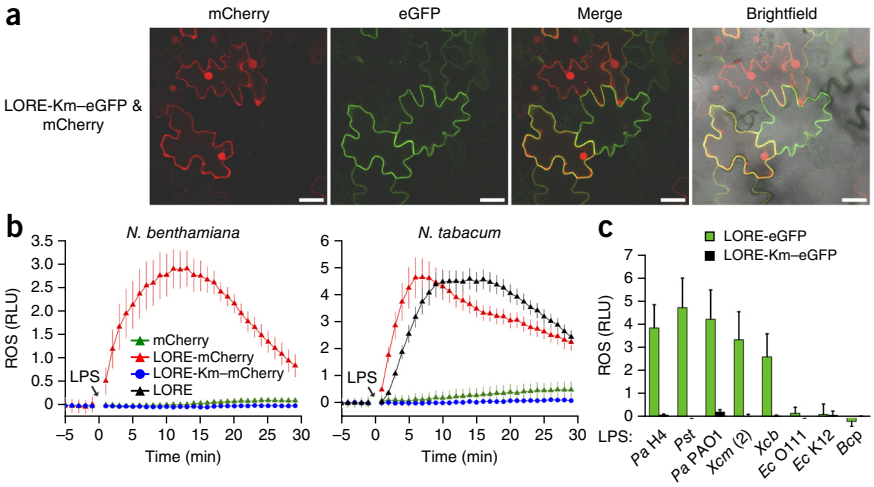

Figure 5 Transient expression of LORE in tobacco. (**a**) Confocal microscopy of epidermal cells from *N. tabacum* transformed to transiently express a fusion of a LORE variant with substitution in the kinase domain (LORE-Km) and eGFP (LORE-Km–eGFP) and to coexpress mCherry (cytoplasmic and nucleoplasmic control). Two additional experiments with *N. benthamiana* expressing LORE-eGFP and mCherry produced similar results (data not shown). Scale bars, 30 µm. (**b**) Accumulation of ROS in leaf discs from *N. benthamiana* or *N. tabacum* transiently transformed with various fusion constructs of mCherry and/or LORE and treated with LPS (20 µg/ml) from *P. aeruginosa* H4. (**c**) Accumulation of ROS in leaf discs from *N. benthamiana* transiently transformed with LORE-eGFP or LORE-Km–eGFP (each infiltrated into one half of the same leaf), assessed 10 min after treatment with various preparations of LPS (25 µg/ml). Data are from one experiment representative of three experiments (**a**) or are representative of three experiments with similar results (**b**,**c**; mean and s.e.m. of $n = 12$ discs from three plants per construct (**b**) or $n = 8$ discs from four plants per construct).

(**Fig. 4a–c**). The elevation in $[Ca^{2+}]_{cyt}$ triggered by *X. campestris* LPS was greatly diminished in *A. thaliana* mutants *lore-1*, *lore-2* and *sd1-29*^{AEQ} but was restored in the two complementation lines described above (**Fig. 4d**). Likewise, the production of ROS triggered by *X. campestris* LPS was dependent on LORE (**Fig. 4e**). While pretreatment with LPS from *P. aeruginosa* H4 or *Pst* DC3000 led to resistance to subsequent infection with *Pst* DC3000 in Col-0 and Col-0^{AEQ} control plants, after pretreatment with LPS from *X. campestris*, we observed only a tendency toward higher resistance to infection with *Pst* DC3000 (**Supplementary Fig. 4b,c**). Pretreatment with LPS from *E. coli* also induced resistance to infection with *Pst* DC3000 in several experiments (**Fig. 4f** and **Supplementary Fig. 4b,c**). However, in contrast to the resistance induced by *Pseudomonas* LPS, the resistance induced by *E. coli* LPS was independent of LORE (**Fig. 4f** and **Supplementary Fig. 4b,c**). Together this suggested that a pattern common to LPS from not only *Pseudomonas* species but also *X. campestris*, but different in LPS from *E. coli*, *S. enterica* and *Burkholderia* species, was specifically sensed in a LORE-dependent manner by *A. thaliana*.

LORE-mediated sensing of LPS is limited to Brassicaceae

SD-RLKs are widespread in land plants, especially in flowering plants (angiosperms)[27]. LORE belongs to an *A. thaliana* SD-RLK subgroup with great 'expansion' in the number of its members compared with the number of members in the corresponding rice SD-RLK subgroup[25,27]. Further phylogenetic analysis of LORE-related proteins of diverse plant families suggested that the sensing of LPS via this class of RLKs is restricted to the family Brassicaceae (**Supplementary Fig. 5a**). Genome analysis of the Brassicaceae species *Eutrema salsugineum* and *Capsella rubella* revealed LORE homologs with 81% and 92% overall amino acid identity, respectively, with *A. thaliana* LORE. Indeed, both species reproducibly generated ROS in response to LPS from *Pseudomonas* species and from *X. campestris* but not in response to LPS from *E. coli* (**Supplementary Fig. 5b**). Thus, the finding that the LPS-sensing specificity of *E. salsugineum* and *C. rubella* was similar to that of *A. thaliana* LORE suggested that the closest homologs described above might be orthologs of LORE that mediate the sensing of LPS.

LORE confers LPS sensitivity onto solanaceous tobacco

According to the phylogenetic analysis, no LORE orthologs would be predicted to exist in solanaceous tobacco. Therefore, we assessed the response of tobacco to LPS. The LPS preparations assessed did not consistently trigger ROS production in leaf discs from *Nicotiana tabacum* or *Nicotiana benthamiana* (**Supplementary Fig. 5c**). Other than slight and variable elevations in resting $[Ca^{2+}]_{cyt}$ after treatment with some of the LPS preparations, we did not observe the typical kinetics for MAMP-triggered elevations in $[Ca^{2+}]_{cyt}$ in *N. tabacum* seedlings (**Supplementary Fig. 5d,e**). Thus, we investigated whether transient expression of LORE in tobacco would be able to confer responsiveness to LPS. In support of the predicted localization, we detected transiently expressed fusions of LORE and enhanced green fluorescent protein (LORE-eGFP) at the plasma membrane in tobacco (**Fig. 5a** and **Supplementary Fig. 5f**). Furthermore, LORE expression in tobacco led to rapid production of ROS induced by *Pseudomonas* LPS, in contrast to results obtained for control tobacco leaves expressing only the red fluorescent protein mCherry or a LORE variant with a mutation in the sequence encoding the conserved ATP-binding site of the kinase domain (**Fig. 5b**). The last result supported the proposal that *LORE* encodes a functional kinase[28] whose activity is required for LPS-triggered accumulation of ROS. In addition to LPS from diverse *Pseudomonas* species, LPS from *X. campestris* elicited ROS production in tobacco expressing LORE-eGFP, but LPS from *B. cepacia* or *E. coli* did not (**Fig. 5c**). Hence, the specificity of LORE-mediated sensing of LPS was maintained upon heterologous expression in tobacco. Together our data showed that functional LORE was sufficient for conferring sensitivity to LPS from *Pseudomonas* and *X. campestris* onto plants.

Lipid A is sufficient for LORE-mediated sensing of LPS

Further investigation of the MAMP-active LPS motif by genetic modification of the LPS-biosynthesis pathway is not feasible in *Pseudomonas* because deep-rough mutations affecting the inner core are lethal[22]. Therefore, we used rough-type LPS from *P. aeruginosa* H4 to further analyze the MAMP activity of the distinct LPS moieties after chemical modification and separation. Treatment with hydrazine removes all ester-bound fatty acids and results in LPS-OH carrying only the two primary amide-bound fatty acids attached to the lipid A backbone. LPS-OH did not trigger an elevation in $[Ca^{2+}]_{cyt}$ or accumulation of ROS in Col-0^{AEQ} plants or Col-0 plants, respectively (**Fig. 6a,b**). Mild acid hydrolysis separates the lipid A moiety from the inner core oligosaccharide[23]. Further fractionation by gel-permeation chromatography and high-performance liquid chromatography

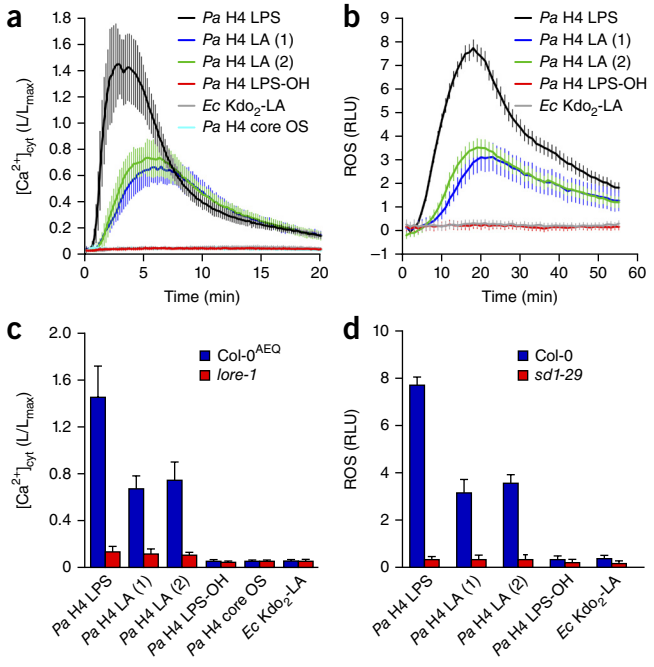

Figure 6 *A. thaliana* PTI responses to various part structures of *Pseudomonas* LPS. (**a**) $[Ca^{2+}]_{cyt}$ in Col-0^{AEQ} *A. thaliana* seedlings treated with whole or various fragments of LPS (25 µg/ml) from *P. aeruginosa* H4 (LPS, whole LPS; LA (1) and LA (2), HPLC lipid A fractions 1 and 2; LPS-OH, hydrazine-treated LPS with only the two amide-bound fatty acids; core OS, isolated core oligosaccharide), or HPLC-purified Kdo_2–lipid A from *E. coli* strain KPM 53 (Ec Kdo_2-LA), with reconstitution of aequorin with 10 µM coelenterazine-h. (**b**) ROS accumulation in Col-0 leaf discs treated with LPS as in **a**. (**c**) Maximum $[Ca^{2+}]_{cyt}$ in *lore-1* and Col-0^{AEQ} seedlings after treatment with LPS as in **a**, with reconstitution of aequorin with 10 µM coelenterazine-h. (**d**) Maximum accumulation of ROS in leaf discs of Col-0 and *sd1-29 A. thaliana* plants treated with LPS as in **a**. Data are pooled from two experiments (**a**,**c**; mean and s.d. of $n = 25, 11, 13, 13, 12$ or 4 (top down) plants per treatment (**a**) or $n = 25, 25; 11, 13; 13, 13; 13, 13; 12, 13;$ or 4, 4 (left to right) plants per genotype and treatment (**c**)) or are pooled from two other experiments (**b**,**d**; mean and s.e.m. of $n = 24, 12, 12, 12$ or 12 discs from twelve plants (top down) (**b**) or $n = 24, 24; 12, 12; 12, 12; 12, 12;$ or 12, 12 (left to right) discs from twelve plants per genotype and treatment (**d**)) with one pair of experiments for both **a** and **c** (together), and another pair for both **b** and **d** (together).

(HPLC) yielded pure core oligosaccharide and lipid A, respectively. We obtained two major lipid A fractions that contained mainly penta-acylated lipid A species with marginal modifications in their fatty acid composition (**Supplementary Fig. 6a**,**b**). Strikingly, these lipid A fractions both elicited elevations in $[Ca^{2+}]_{cyt}$ and production of ROS, albeit to a lower extent than did rough-type LPS, whereas the isolated core oligosaccharide did not induce elevations in $[Ca^{2+}]_{cyt}$ (**Fig. 6a**,**b**). Moreover, the lipid A–induced elevations in $[Ca^{2+}]_{cyt}$ were dependent on LORE, as they were diminished in *A. thaliana* mutant *lore-1* but were restored in the complementation lines described above (**Fig. 6c** and **Supplementary Fig. 6c**). Likewise, lipid A–induced production of ROS required LORE (**Fig. 6d**). In agreement with the results obtained with *E. coli* LPS, HPLC-purified Kdo_2–lipid A (hexa-acylated) from the deep-rough *E. coli* strain KPM 53 (ref. 29) did not trigger elevations in $[Ca^{2+}]_{cyt}$ or production of ROS (**Fig. 6a**,**b**). These results demonstrated that in *A. thaliana*, *Pseudomonas* LPS was sensed specifically through its lipid A moiety and that the lipid A moiety was sufficient for immunological sensing in a LORE-dependent manner.

DISCUSSION

LPS preparations from various bacteria induce defense responses in many plant species, which suggests that plants have LPS-sensing systems with species-specific recognition of distinct structural determinants[14]. So far, however, the LPS-sensing components in plants have remained unknown. In this study, we have provided evidence of a sensitive system for sensing *Pseudomonas* LPS in the plant family Brassicaceae. Smooth- and rough-type LPS as well as isolated lipid A acted as MAMPs and elicited PTI in *A. thaliana* via LORE, a B-type lectin SD-RLK. LPS from *X. campestris* was likewise sensed in a LORE-dependent manner, whereas LPS from *E. coli*, *S. enterica* or *Burkholderia* species did not activate typical early PTI responses in *A. thaliana*. In mammals, TLR4-MD2 is strongly activated by the prototypical enterobacterial hexa-acylated lipid A of *E. coli*[30], while other lipid A variants from diverse bacteria, including *Pseudomonas* species, are less potent agonists due to differences in acylation, phosphorylation and other structural modifications[30,31]. Penta-acylated lipid A from *P. aeruginosa* activates human TLR4 only weakly but strongly activates mouse TLR4, which demonstrates host species specificity in the sensing of lipid A[8,31]. Similarly, *A. thaliana* seemed to sense a specific structural LPS variant present in LPS from both *Pseudomonas* species and *X. campestris*. The lipid A part was indispensable, because neither the isolated core oligosaccharide nor LPS with only two fatty acid residues (LPS-OH) triggered elevations in $[Ca^{2+}]_{cyt}$. Indeed, two HPLC-purified *Pseudomonas* lipid A fractions containing mainly penta-acylated lipid A were sufficient to trigger elevations in $[Ca^{2+}]_{cyt}$ in *A. thaliana* in a LORE-dependent manner. Similarly, isolated *X. campestris* lipid A induces the expression of genes encoding defense-related proteins in *A. thaliana*[17]. We used the same concentration (weight per volume) of LPS and lipid A in our experiments; however, although the intact LPS has a higher molecular weight than the isolated lipid A, we observed that the elevations in $[Ca^{2+}]_{cyt}$ triggered by *Pseudomonas* lipid A were lower than those induced by the respective rough-type LPS. Hence, we conclude that the presence of the core oligosaccharide in the LPS molecule enhanced the sensitivity of *A. thaliana* to LPS. The inner core of *Pseudomonas* LPS, which is highly negatively charged due to phosphorylation[8,22], might contribute to its greater activity than that of isolated lipid A, as it might promote interactions with receptors through specific ionic interactions. Along this line, the core oligosaccharide of *Capnocytophaga canimorsus* LPS has been shown to boost its binding to mammalian MD2 (refs. 32,33). Similarly, substitution of the lipid A phosphate groups with phosphoethanolamine as well as de-phosphorylation of *X. campestris* LPS impair its ability to induce the expression of genes encoding defense-related proteins in *A. thaliana*[17,34]. Since LORE also sensed LPS from the human pathogen *P. aeruginosa*, mechanistic insights into this sensing of LPS by plants opens new perspectives for the development of antibacterial therapies to combat bacterial infection or sepsis-related complications.

A. thaliana plants with loss-of-function mutations in the gene encoding LORE had considerably impaired PTI responses after being elicited by LPS from several *Pseudomonas* species and *X. campestris* strains, compared with the PTI responses of control plants. However, individual LPS preparations caused a slight response in the *A. thaliana* mutants *lore-1*, *lore-2* and *sd1-29* as well as in tobacco. Pretreatment with *E. coli* LPS enhanced resistance to bacterial infection in *A. thaliana*, but apparently this LPS did not trigger the typical early signaling reactions consistently observed with *Pseudomonas* LPS and other MAMPs. These LORE-independent responses may have been due to low levels of contaminants. Alternatively, there may be another LPS-sensing system (or other such systems), possibly with low sensitivity, or direct

incorporation of LPS into membranes via its lipid A moiety may initiate 'noncanonical' recognition of LPS[35,36]. Such different sensing systems might account for the discrepancies between our analyses and published studies (as well as among those studies) reporting immune responses to LPS of different origin, including LPS from *E. coli* and *B. cepacia*, in *A. thaliana* and other plant species[14,16,19]. We consistently observed rapid and specific elevations in $[Ca^{2+}]_{cyt}$ and other typical PRR-mediated PTI reactions in *A. thaliana* in response to all the LPS tested from *Pseudomonas* species and *X. campestris*, as well as in response to HPLC-purified lipid A from *P. aeruginosa*. Related species such as *C. rubella* and *E. salsugineum* that have LORE homologs also rapidly produced ROS upon treatment with LPS from *Pseudomonas* species or *X. campestris* but not upon treatment with LPS from *E. coli*. Together our data suggest that LORE is an immunoreceptor for LPS from *Pseudomonas* and structurally related LPS. Additional studies should determine whether LPS binds directly to LORE and if accessory LPS-binding proteins, potentially analogous to the mammalian sensing of LPS[12,37], are required. However, since heterologous LORE expression in *N. benthamiana* led to sensitivity to LPS with the same specificity as that in *A. thaliana*, this would indicate a key role for LORE in the binding of LPS.

LORE is also notable in terms of evolutionary aspects of eukaryotic sensing of MAMPs. Although mammalian TLR5 and plant FLS2 flagellin receptors both contain LRR ectodomains, they evolved independently and recognize distinct flagellin epitopes[2,38]. In contrast, mammals and plants evolved structurally different receptor types for the sensing of LPS; i.e., an LRR ectodomain for TLR4 (ref. 37) but a modular ectodomain containing a plant-specific B-type lectin domain for LORE[25]. Strikingly, although the lipid A moiety is sufficient to confer immunological recognition in plants and animals, they differ in their 'preference' for detecting distinct acylation patterns. Additional research should determine if other phytopathogenic bacteria are sensed through their lipid A moiety and if this requires LORE. Future studies may identify the different selective factors driving the evolution of distinct lipid A–sensing preferences in mammals versus plants.

The term 'S-domain' (SD) originates from the role of plant B-type lectin RLKs in self-incompatibility in the mating of *Brassica* plants[27,39,40], a process that can be described as an endogenous defense strategy to prevent inbreeding. However, LORE is not closely related to *Brassica* S-locus receptor kinases. Since SD-RLKs are widespread in the plant kingdom, they probably fulfill diverse functions[27]. Several genes encoding SD-RLKs in *A. thaliana* are induced by biotic stress, which suggests that other SD-RLKs might be involved in innate immunity[25]. Given the common occurrence of SD-RLKs in land plants, downstream signaling components may be sufficiently conserved to allow interfamily receptor transfer. As proof of concept, we conferred the sensing of LPS via LORE onto tobacco. Thus, transfer of LORE to crops such as tomatoes, potatoes or cereals might enable the engineering of traits for resistance to bacteria such as *Pseudomonas* and *Xanthomonas* species, which include major plant pathogens.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.R., M.S., T.I., L.W. and J.L. performed experiments; S.R., Y.A.K., P.M.S.-C. and U.Z. prepared LPS; S.R. and D.S. initiated the project; S.R. designed plant experiments; N.G. and U.Z. designed LPS purification and chemical dissection experiments; S.R., N.G. and J.L. analyzed the data; S.R., N.G., J.L. and R.H. interpreted data; S.R., N.G., R.H., U.Z., J.L. and D.S. wrote the manuscript; and all authors discussed the results and approved of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Plant material and growth conditions. Col-0 *A. thaliana* expressing apoaequorin under control of the cauliflower mosaic virus 35S (CaMV35S) promoter in the cytosol (Col-0^{AEQ}) was obtained from M. Knight[41]. T-DNA insertion lines *cerk1-2* (*lyk1*; TAIR accession code At3G21630; NASC accession code GK-096F09) and *lyk4* (TAIR accession code At2G23770; NASC accession code WiscDsLox297300_01C) on the Col-0^{AEQ} background were provided by G. Stacey[6]. The mutants *fls2-26* (TAIR accession code At5G46330), *efr-1* (TAIR accession code At5G20480; NASC accession code SALK_044334), *bak1-4* (TAIR accession code At4G33430; NASC accession code SALK_116202) in the Col-0^{AEQ} background, *rbohD* (TAIR accession code At5G47910; dSpm transposon mutant), *mpk3-1* (TAIR accession code At3g45640; NASC accession code SALK_151594) and *mpk6-3* (TAIR accession code At2G43790, NASC accession code SALK_127507) have been described[20,24]. The mutants *sd1-29* (TAIR accession code At1G61380; NASC accession code SAIL_857_E06) and *sd1-23* (TAIR accession code At1G61390; NASC accession code GK-079G10) were from the European Arabidopsis Stock Centre and were genotyped by standard methods (primers, **Supplementary Table 2**). The mutant *sd1-29*^{AEQ} was generated by crossing of *sd1-29* and Col-0^{AEQ} plants. For growth in liquid medium, *A. thaliana* seeds were 'surface-sterilized'[24], were stratified for 2 d or more at 4 °C and were grown in 24-well plates (~15 seedlings per well) under long-day conditions (16 h of light) at 20–22 °C in liquid MS medium (0.5× Murashige & Skoog medium, including vitamins (Duchefa), 0.25% sucrose, 1 mM MES, pH 5.7). Experimental *A. thaliana* and *C. rubella* (NASC accession code N22697; from M. Quint) were grown on soil in climate chambers with 8 h of light; *E. salsugineum* ecotype Shandong plants (obtained from E. Glawischnig), *N. tabacum* var. Samsun and *N. benthamiana* were grown with 10 h of light at 20–22 °C.

MAMPs. The peptides flg22 (QRLSTGSRINSAKDDAAGLQIA)[42] and elf18 (ac-SKEKFERTKPHVNVGTIG)[43] were synthesized on an Abimed EPS221 system (Abimed). Shrimp shell chitin (C9752; Sigma) was ground into a fine powder and resuspended in water, and the soluble fraction was used for experiments. LPS preparations are summarized in **Supplementary Table 1**. LPS preparations have been described or were donated (**Supplementary Table 1**), and unpublished LPS preparations are described below. These LPS preparations were digested overnight at 50 °C with proteinase K (2 mg/ml LPS; 10 µg/ml proteinase K) prior to use in bioassays. LPS from *E. coli* strains K12 and O111:B4, as well as *S. enterica* serovars Thypimurium and Minnesota R595 was obtained from InvivoGen or Enzo Life Sciences.

For the preparation of LPS from *Pseudomonas syringae* pv. *tomato* DC3000, bacteria were grown in King`s B medium at 26 °C under shaking (230 rpm) to an absorbance of ~0.8 at 600 nm, then were harvested by centrifugation (3,000*g*) for 20 min at 4 °C, then washed with ethanol, acetone (twice) and diethyl ether, and then dried (recovery, 10.79 g). For hot phenol-water extraction (PW extraction)[44], bacteria were stirred for 20 min at 68 °C in 45% aqueous phenol (18.5 ml per g bacteria). After centrifugation (5,500*g*) for 30 min at 4 °C, the upper water phase was collected (~60 ml). The extraction was repeated with the same volume of water. Combined water phases and the phenolic phase were dialyzed against water at 4 °C (12- to 16-kDa cutoff; Biomol) and lyophilized. In contrast to results obtained by the usual PW extraction, the water phase was found to contain almost no LPS. Therefore, a phenol–chloroform–petroleum ether extraction[45] was performed with the material of the phenolic phase (7.1 g). It was resuspended in phenol(90%)–chloroform–petroleum ether (boiling range, 40–60 °C) (2:5:8 (vol/vol/vol); 80 mg/ml)) with an Ultra-Turrax, then the suspension was stirred for 30 min at room temperature and centrifuged (5,800*g*) for 20 min at 20 °C. The supernatant was collected and the extraction was repeated twice. Combined supernatants were evaporated in vacuum until phenol crystallization began. LPS was precipitated overnight at 4 °C with 11.8 ml water and for 3 d at 4 °C with 68 ml ethanol. After centrifugation (3,000*g*) for 20 min at 4 °C, the precipitate was washed three times with acetone (5–10 ml) at 3,000*g* for 15 min at 4 °C and was dried. LPS (14.6 mg) was resuspended in water (5 mg/ml) and was sequentially treated overnight at 37 °C with DNase and RNase (~10 µg/ml) and overnight at 50 °C with proteinase K (~10 µg/ml).

P. syringe pv. *apii* (bacterial mass provided by K. Rudolph) and *P. alcaligenes* 537 (bacterial mass provided by W. Bitter) were isolated by hot PW extraction as described above with the following modifications. For *P. syringe* pv. *apii*, 9.5 ml 45% aqueous phenol was used per gram dried bacterial mass (centrifugation: 2,800*g* for 20 min at 0 °C). Three extraction steps were performed to gain acceptable yields. The water phase of the third extraction was processed separately and contained ~80% of the total LPS. After dialysis for 4 d at 4 °C and lyophilization, the pellet was resuspended in water (55 mg/ml) and was ultracentrifuged three times (~200,000*g*) for 4 h at 4 °C. The LPS pellet (10 mg/ml in water) was sequentially treated overnight at room temperature with DNase, RNase and proteinase K (each at ~60 µg/ml), then underwent dialysis and lyophilization. For *P. alcaligenes* 537, enzymatic treatment (enzymes at ~35 µg/ml) was performed prior to PW extraction. After enzymatic treatment, samples were dialyzed for 5 d at 4 °C and then centrifuged (14,300*g*) for 20 min at 0 °C, and the pellet was washed twice with acetone. LPS was extracted with 14 ml of 45% aqueous phenol per gram dried bacterial mass (centrifugation: 4,300*g* for 30 min at 4 °C) and the combined water phases underwent dialysis and lyophilization.

E. coli KPM 53 (ref. 29) was grown overnight at 37 °C. Cells were washed for 15 min at 4 °C (18,000*g*), twice with ethanol and acetone and once with diethyl ether. The dried pellet was resuspended in water (30 mg/ml), sequentially treated overnight at room temperature with DNase and RNase and proteinase K (each at ~30 µg/ml), dialyzed for 2 d at 4 °C against water and lyophilized. LPS was extracted by phenol–chloroform–petroleum ether extraction[45]. The pellet was suspended in phenol (90%)–chloroform–petroleum ether (boiling range, 40–60 °C) (2:5:8 (vol/vol/vol); 120 mg/ml) with an Ultra-Turrax, then the mixture was stirred for 30 min at room temperature and the supernatant was collected after centrifugation (5,800*g*) for 20 min at 4 °C. The extraction was repeated three times, then the supernatants were combined and evaporated in vacuum (50 °C) until phenol crystallization began. LPS was precipitated in the cold by the addition of water (phenol concentration, >80%), then samples were centrifuged (5,800*g*) for 20 min at 25 °C and then washed two times with 80% phenol and three times with acetone. The triethylamine salt of the LPS was formed[46] and purified by reversed-phase HPLC as described[32] with the following modifications: the initial solvent system (2% B) was maintained for 20 min, followed by a linear two-step gradient raising from 2% to 17% B (20–50 min) and 17% to 27% B (50–85 min). The solvent was held for 25 min at 27% B, then the column was re-equilibrated for 10 min to 2% B and was held there for 10 min before the next injection. The pool used for the experiments contained solely hexa-acylated LPS with a monoisotopic molecular mass of 2237.337 Da (2× GlcN, 2× *P*, 2×Kdo, 4× 14:0 (3-OH), 1× 14:0, 1× 12:0). For the injection (sample concentration, 8 mg/ml), methanol-chloroform-water (57:12:31 (vol/vol/vol)) containing 10 mM NH_4OAc and 0.1 M Na-EDTA, pH 7 (4:1 (vol/vol))was used.

For the preparation of lipid A and core oligosaccharide of *P. aeruginosa* H4 by acid hydrolysis, LPS from *P. aeruginosa* H4 (104.7 mg) was dissolved in 14 ml water, 1.75 ml of a solution of 10% SDS, and 1.75 ml acetate buffer (1 M NaOAc, pH 4.4), then the mixture was heated for 3 h at 100 °C with occasional mixing and lyophilized. SDS was removed by four washes with 60 ml 2 M HCl/ethanol (1:99 (vol/vol) (5,800*g* for 20 min at 20 °C). The dried pellet was resuspended in 5 ml water. Afterward, 5 ml $CHCl_3$ and CH_3OH (4:1 (vol/vol)) were added and the suspension was mixed vigorously and centrifuged (5,800*g*) for 10 min at 4 °C. The organic phase was collected and then the water phase (including the interphase) was extracted again successively with 5 ml $CHCl_3$ and CH_3OH (9:1 (vol/vol)) and $CHCl_3$. All organic phases (containing lipid A) were combined, evaporated in vacuum and dried (recovery, 21.5 mg). The core oligosaccharide–containing water phase was lyophilized. Core oligosaccharides were purified by gel-permeation chromatography on a Sephadex G-50 column (2.5 × 80 cm; GE Healthcare) through the use of pyridine–acetic acid–water (8:20:2,000 (vol/vol), pH ~4.7) as eluent. Oligosaccharides were monitored by a Knauer differential refractometer. Fractions containing the core sugars (detected as per-acetylated methyl glycosides by gas-liquid chromatography–mass spectometry) were collected and lyophilized. In addition to the pure core oligosaccharide (10.3 mg), a second pool containing the core oligosaccharide that eluted earlier was obtained (15.9 mg), in which a polyamine of an as-yet-unknown structure was present. Only the polyamine-free core oligosaccharide was used for biological experiments. The lipid A was further purified by reversed-phase HPLC (**Supplementary Figure 6**) essentially as described[32].

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For preparation of the core-lipid A backbone oligosaccharide with amide-bound fatty acids (LPS-OH), LPS (50 mg) from *P. aeruginosa* H4 was de-*O*-acylated by mild hydrazinolysis (incubation for 2 h at 37 °C in 2.0 ml hydrazine). The product was precipitated for 30 min at −20 °C from ice-cold acetone (20 ml), then centrifuged (3,800*g*) for 20 min at 4 °C, washed four times with ice-cold acetone (3,800*g* for 20 min at 4 °C) and, finally, centrifuged (47,800*g*) for 30 min at 4 °C. The dried pellet was resuspended in water (20 mg/ml) and the mixture was acidified to a pH of 5–6 with 10 µl of 4 M HCl. Acetone (−20 °C) was added, the sample was centrifuged (47,800*g*) for 30 min at 4 °C and the pellet was washed once with acetone and dried (26.8 mg LPS-OH).

***A. thaliana* mutant screen.** The Col-0^{AEQ} mutant population and screening procedure has been described in detail[24]. Col-0^{AEQ} seeds underwent mutagenesis for 8 h at room temperature with subtle shaking in 0.4% ethyl methane sulfonate, then were washed with water and dried. M_1 plants were grown on soil in a greenhouse, and seeds were harvested from individual M_1 plants. M_2 seedlings (12 or more per M_1 plant line) were grown in sterile MS medium, then were transferred to individual wells of 96-well plates and reconstituted overnight in the dark with 10 µM coelenterazine-h. Aequorin luminescence was measured as described below but without discharge. Seedlings with a much lower elevation in aequorin luminescence upon application of 5 µg/ml LPS from *P. aeruginosa* H4 than that of control Col-0^{AEQ} seedlings were 'rescued' from the 96-well plates and were grown on soil for seed setting. The phenotype of putative mutants was verified by analysis of the corresponding M_3 offspring with quantitative $[Ca^{2+}]_{cyt}$ measurements as described below by estimation of L_{max}. We obtained four mutants (*A. thaliana* mutants *lore-1* through *lore-4*) that were insensitive to *Pseudomonas* LPS and were allelic in a complementation test; i.e., F_1 hybrids resulting from reciprocal crosses failed to complement each other for LPS-induced elevations in $[Ca^{2+}]_{cyt}$.

Mapping and sequencing of candidate genes. Mapping populations were generated by the crossing of *A. thaliana lore-1* or *A. thaliana lore-4* mutant lines with the *A. thaliana* ecotype Landsberg *erecta*-0 (L*er*-0). M_2 offspring seedlings of these crosses with much lower elevations in aequorin luminescence upon application of 5 µg/ml LPS from *P. aeruginosa* H4 were 'rescued' and were grown on soil for seed setting. This resulted in 134 plants for the *lore-1* × L*er*-0 cross, 82 for the *lore-4* × L*er*-0 cross and 101 for the *lore-4* × L*er*-0^{AEQ} cross. The analysis of PCR-based markers localized *LORE* between the insertion-deletion markers 469696 and CER460336 (ref. 47) on chromosome 1. For sequence analysis, *LORE* was amplified from genomic DNA by PCR with gene-specific primers SD1-29-F and SD1-29-R with Phusion Hot Start High-Fidelity DNA Polymerase (Thermo Scientific), and purified PCR products were sequenced with the appropriate primers (**Supplementary Table 2**). *LORE* of *A. thaliana* mutants *lore-1* through *lore-4* was sequenced as described above, and a single-nucleotide exchange was detected in each mutant. The independently obtained T-DNA insertion line of SD1-29 (see above) was found to be allelic to all four mutants in a complementation test; i.e., F_1 hybrids resulting from reciprocal crosses failed to complement each other for LPS-induced elevations in $[Ca^{2+}]_{cyt}$.

Molecular cloning and generation of transgenic lines. Full-length *SD1-29* coding sequence was amplified by PCR with primers SD129-START, SD129-STOP, SD129-EspMut-F and SD129-EspMut-R (to mutate the internal Esp3I site) (primers, **Supplementary Table 2**) and was cloned into pUC18 modified for GoldenGate-based cloning by procedure similar to that described[48]. Site-directed mutagenesis (for LORE-Km, with substitution of alanine for the lysine at position 516) was performed by whole-plasmid amplification with Phusion Hot Start High-Fidelity DNA polymerase (Thermo Scientific) and subsequent recirculation by GoldenGate methods. GoldenGate techniques were used to combine coding sequences with a CaMV35S promoter and terminator and sequence encoding a carboxy-terminal eGFP or mCherry epitope tag with a ten-glycine linker. Expression cassettes were transferred into GoldenGate-modified binary plasmid pCB302. For complementation of the mutant *lore-1*, a genomic *SD1-29* fragment including the putative promoter was amplified by PCR with primers SD129-PROM, SD129-STOP, SD129-EspMut-F and SD129-EspMut-R (primers, **Supplementary Table 2**) and was cloned into GoldenGate-modified pCB302. After transfer of the constructs into *Agrobacterium tumefaciens* strain GV3101, *A. thaliana* plants were transformed by floral-dip transformation. Transgenic plants were selected through the use of spraying with BASTA (glufosinat-ammonium; Bayer).

Aequorin luminescence measurement. For measurement of aequorin luminescence, 8- to 10-day-old liquid-grown apoaequorin-expressing seedlings were placed individually in 96-well plates in coelenterazine and water (native coelenterazine or coelenterazine-h; PJK.) and were incubated in the dark overnight. Luminescence was recorded by scanning of one row at intervals of 6 s or two rows at intervals of 10 s, with a Luminoskan Ascent 2.1 luminometer (Thermo Scientific) or a Tecan F200 luminometer (Tecan). The remaining aequorin was discharged by the addition of 150 µl of 2 M $CaCl_2$ with 20% ethanol per well. $[Ca^{2+}]_{cyt}$ concentrations were calculated as L/L_{max} (luminescence counts per second relative to total luminescence counts remaining) as described[24].

Detection of ROS in *A. thaliana* leaves. For detection of ROS, leaf discs 3 mm in diameter from 6- to 8-week-old soil-grown plants were floated overnight in 200 µl water in 96-well plates in the dark. Shortly before the assay, water was replaced with 100 µl of 5 µM L-012 (WAKO Chemicals) and 2 µg/ml horseradish peroxidase (type II; Roche). Luminescence was recorded as relative light units (RLU) at 1-minute intervals (Tecan F200). After 10 min of background reading, elicitors were added to the appropriate final concentration, and luminescence readings continued over 60 min. Results were normalized to average ROS at 5 min before treatment with LPS, followed by subtraction of results for untreated controls (included for each genotype on the same plate).

Immunoblot analysis of MAPK activation. 14-day-old liquid-grown seedlings were equilibrated for 24 h in fresh MS medium. Medium was discarded and after 30 min of recovery, MAMPs in MS medium were added at the appropriate concentration. Seedlings were harvested at the appropriate time points. Protein extraction and immunoblot with antibody to phosphorylated MAPKs (p44, p42, and Erk; 9101; Cell Signaling Technology) were performed as described[20].

Gene-expression analysis. 12-day-old liquid-grown seedlings were equilibrated in fresh MS medium for 2 d before treatment with the appropriate elicitors in MS medium or with medium as a control. Whole seedlings were harvested at 0 h or 4 h and were frozen in liquid nitrogen. Total RNA was extracted by the conventional Trizol method, followed by treatment with DNase I (Thermo Scientific) and reverse-transcription with oligo$(dT)_{18}$ and RevertAid reverse transcriptase according to the manufacturers' instructions (Thermo Scientific). Gene expression was analyzed by semi-quantitative PCR (primers, **Supplementary Table 2**). The cDNA was diluted 1:10 in water and a 2-µl aliquot was used for standard PCR in a volume of 20 µl. Amplification conditions were as follows: 1 min at 95 °C, then 23–44 cycles of 10 s at 95 °C, 10 s at 54 °C and 45 s at 72 °C, followed by 5 min at 72 °C. The optimal number of PCR cycles determined to be in the non-saturated range has been established for each primer pair (**Supplementary Table 2**). PCR products were separated by electrophoreses through 1% TBE agarose gels containing ethidium bromide. Quantitative real-time PCR was performed with Maxima SYBR Green-ROX qPCR Master Mix (Thermo Scientific) and a MxPro3005P according to manufacturer's instructions (Agilent Technologies) as follows: 40 cycles of 30 s at 95 °C, 60 s at 60 °C and 25 s at 72 °C. Results were analyzed with MxPro-Mx3005P Software (Agilent). The expression of *SD1-29* was normalized to that of the gene encoding ubiquitin. *SD1-29* expression in mutant lines is presented relative to the *SD1-29* expression of the Col-0 control. Amplification specificity was analyzed by non-template controls and primer-dissociation curves. All primer pairs used for gene-expression analysis were chosen to span at least one intron if possible.

Histochemical staining of callose. 10-day-old liquid-grown seedlings were equilibrated in fresh MS medium 24 h before application of LPS in MS medium or of medium as a control. After 24 h of incubation under light, seedlings were cleared overnight in 95% ethanol, then were washed with 80% ethanol, twice with 50% ethanol and twice with water and were equilibrated

for 1 h in 100 mM Na_2HPO_4 (pH 9). Seedlings were stained for 1 h in the dark with 0.01% aniline blue in 100 mM Na_2HPO_4 (pH 9) and fluorescence was examined with a Zeiss Axioplan epifluorescence microscope.

Bacterial infection assay. For spray inoculation, leaves of 6-week-old plants were inoculated by being sprayed with a suspension of 1×10^8 CFU/ml of *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) containing 0.04% Silwet-L77 (Lehle Seeds). Infected leaves were harvested 3–4 h (day 0) and 3 d after inoculation. Bacterial growth was determined by counting of colonies by serial dilution on LB plates supplemented with 75 μg/ml rifampicin as described[26]. Statistical analysis (genotype × treatment; two-way ANOVA) was performed with GraphPad Prism 5.0.

For pressure infiltration, 25 μg/ml LPS or water (control) was infiltrated into *A. thaliana* leaves with a needle-less syringe. After 24 h, *Pst* DC3000 was infiltrated into the leaves at a dose of 1×10^5 CFU/ml. Bacterial growth was assessed as described above. Statistical analysis (one-way ANOVA) was performed with GraphPad Prism 5.0.

Transient expression in tobacco and confocal microscopy. Overnight cultures (supplemented with 100 μM acetosyringone) of *A. tumefaciens* strain GV3101 carrying the appropriate construct(s) were harvested by centrifugation (1,000*g*) for 5 min at room temperature and were washed twice with infiltration medium (10 mM $MgCl_2$, 10 mM MES, pH 5.7, and 150 μM acetosyringone). Bacteria were resuspended in infiltration medium to an absorbance of 1.0 at 600 nm, then were incubated for 4–6 h at room temperature and were infiltrated via a needle-less syringe into leaves of 6- to 8-week-old tobacco plants. For expression in *N. tabacum*, bacteria were diluted to an absorbance of 0.1 at 600 nm, and for expression in *N. benthamiana*, bacteria (at an absorbance of 0.05 at 600 nm) were mixed at a ratio of 1:1 with *A. tumefaciens* GV3101 carrying the p19 suppressor of silencing[49]. For coexpression, bacteria carrying the appropriate construct(s) were mixed at a ratio of 1:1 prior to infiltration. Leaf discs (3 mm in diameter) were cut 2 d after infiltration and were used for detection of ROS as described above. Fluorescence from eGFP and mCherry was detected with a Leica TCS SP5 confocal microscope with standard settings. Images were processed with Leica LAS AF software.

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