Structural signatures in EPR3 define a unique class of plant carbohydrate receptors

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Receptor-mediated perception of surface-exposed carbohydrates like lipo- and exopolysaccharides (EPS) is important for non-self recognition and responses to microbial associated molecular patterns in mammals and plants. In legumes, EPS are monitored and can either block or promote symbiosis with rhizobia depending on their molecular composition. To establish a deeper understanding of receptors involved in EPS recognition, we determined the structure of the *Lotus japonicus* (*Lotus*) exopolysaccharide receptor 3 (EPR3) ectodomain. EPR3 forms a compact structure built of three putative carbohydrate-binding modules (M1, M2 and LysM3). M1 and M2 have unique $\beta\alpha\beta$ and $\beta\alpha$ folds that have not previously been observed in carbohydrate binding proteins, while LysM3 has a canonical $\beta\alpha\beta$ fold. We demonstrate that this configuration is a structural signature for a ubiquitous class of receptors in the plant kingdom. We show that EPR3 is promiscuous, suggesting that plants can monitor complex microbial communities though this class of receptors.
Nitrogen-fixing symbiosis between legumes and rhizobia is governed by a two-step receptor-mediated recognition mechanism. In the first step, rhizobial lipochitooligosaccharides (LCOs or Nod factors) are perceived by plant LCO receptors, which induces the development of root nodule primordia, the entrapment of rhizobia in root hair curls and triggers the expression of symbiotic genes for bacterial infection, including Epr31-5. The second step controls the subsequent progression of nodule infection and is mediated by the single-pass transmembrane receptor kinase EPR36. Here, we determined the structure of the membrane receptor kinase EPR3 of nodule infection and is mediated by the single-pass transmembrane receptor kinase EPR3. The natural symbiont of Lotus, Mesorhizobium loti (R7A), synthesises EPS polymers built from octasaccharide-repeating units. Monomeric octasaccharides from octasaccharide-repeating units. Monomeric octasaccharides (LCOs or Nod factors) are perceived by plant LCO receptors, which induces the development of root nodule primordia, the entrapment of rhizobia in root hair curls and triggers the expression of symbiotic genes for bacterial infection, including Epr31-5. The second step controls the subsequent progression of nodule infection and is mediated by the single-pass transmembrane receptor kinase EPR36.

In contrast, truncated EPS produced by the epidermal and cortical tissues of Lotus and Medicago roots. Studies of rhizobia and host plant mutants show that EPS perception and subsequent EPR3 signalling promotes infection of the epidermal and cortical tissues of Lotus and Medicago roots1-5,11. In contrast, truncated EPS produced by the exoU mutant strain (R7A exoU EPS) blocks rhizobial infection and colonisation in an EPR3-dependent manner, suggesting that the perception of EPS is an additional compatibility-determining step in legume–rhizobia interactions1,4. Here, we determined the structure of the defining member of a conserved and unique class of plant EPS receptors and show that EPR3 is capable of directly perceiving EPS from different bacterial species, suggesting a broader role in surveillance of microbial communities.

Results and discussion

The crystal structure of EPR3. To understand the basis of EPS perception, the ectodomain of Lotus EPR3 (hereafter referred to as EPR3) was expressed in insect cells and purified for structural studies. Despite numerous attempts, EPR3 did not crystallise. We therefore generated a miniature llama-derived antibody (nanobody) to facilitate crystallisation. We raised an immune response against EPR3 by immunising a llama and selected nanobodies by phage display12,13. The high-affinity nanobody, Nb186, forms a stable complex with EPR3 as demonstrated by a mobility shift in size-exclusion chromatography (SEC) experiments (Supplementary Fig. 1a). The co-purified deglycosylated-EPR3-Nb186 complex was isolated (Supplementary Fig. 1b, c) and crystallised, and the structure was determined and refined to 1.9 Å resolution (Supplementary Fig. 2 and Supplementary Table 1). The overall structure of EPR3 consists of three interconnected modules (M1, M2 and LysM3) arranged in a cloverleaf-shape and stabilised by disulphide bridges (Fig. 1a). The crystal structure of EPR3 reveals an M1 fold that is structurally unique (Fig. 1b). M1

Fig. 1 The crystal structure of EPR3. a Cartoon representation of the EPR3 crystal structure with each of the modules M1, M2 and LysM3 coloured in orange, brown and grey, respectively. Secondary structure elements and disulphide bridges are indicated. The dotted line highlights the unique M1 domain. b-d Individual carbohydrate-binding modules M1, M2 and LysM3 of EPR3 with labels indicating their secondary structures. The panels below show the modules superimposed on the corresponding LysM domains of CERK6 (PDB - 5L52) coloured in light blue.
is composed of only one α-helix and three elongated β-strands. The exterior β2-strand is stabilised by seven backboned hydrogen bonds to the adjacent β3-strand, which gives M1 an overall βαββ arrangement where the three β-strands form an extended anti-parallel β-sheet (Fig. 1b). The M2 domain of EPR3 is also unusual, as it contains a βαβ fold and lacks the defined second α-helix compared with a canonical LysM domain (Fig. 1c). LysM3 has the standard βαβ fold of LysM proteins, with a root-mean-square deviation (RMSD) of 1.2 Å to the LysM3 domain of Lotus chitin receptor CERK614. A DALI search in the Protein Data Bank (PDB) revealed that M1 in EPR3 has no close structural homologues found in dicots (legumes and non-legumes) and monocots showing the conserved βαββ structural arrangement. As it contains a βαββ fold and lacks the defined second α-helix compared with a canonical LysM domain (Fig. 1c), LysM3 has the standard βαβ fold of LysM proteins, with a root-mean-square deviation (RMSD) of 1.2 Å to the LysM3 domain of Lotus chitin receptor CERK614. A DALI search in the Protein Data Bank (PDB) revealed that M1 in EPR3 has no close structural homologues found in dicots (legumes and non-legumes) and monocots showing the conserved βαββ structural arrangement.
the ability of EPR3 to distinguish between EPS of different structures and compositions, we examined ligand binding in solution using microscale thermophoresis (MST) (see all ligands in Supplementary Fig. 6). EPR3 binds the compatible monomeric octasaccharide R7A EPS with an equilibrium-dissociation constant \(K_d\) of \(38.1 \pm 7.5 \mu M\) (Fig. 4a). The binding is neither affected by the glycosylation state of the receptor nor the binding of Nb186 (Supplementary Fig. 7), indicating that the EPR3-Nb186 crystal structure is most likely presented in its biologically active state. To detect if ligand binding affects EPR3 oligomerization as a potential signalling mechanism, we determined the SAXS solution structure of EPR3 saturated with R7A EPS. The scattering data and ab initio reconstructions show that the ligand-bound receptor retains its monomeric state with the same overall structure, dimensions and stem-like arrangement as the ligand-free state (Supplementary Fig. 8; Table 2). This could mean that EPR3 signals as a monomer, or, maybe more likely, associates with as yet unidentified co-receptor(s) to form a ligand-induced signalling complex as known from other single-pass transmembrane receptor kinases (Fig. 3f)\(^{2}\). To further investigate ligand selectivity, we first assessed if EPR3 binds the immune response-inducing chitin polymer (CO6) known to be perceived by canonical LysM receptors\(^{14,18,19}\) and found that EPR3 is unable to bind CO6 (Fig. 4b). Next, we examined the ability of EPR3 to recognise symbiotic signalling LCO molecules using the Bio-layer interferometry methodology previously used to show robust M. loti Nod factor binding to Lotus Nod factor receptors NFR1 and NFR5\(^{20}\). EPR3 binding to LCO was not detected. Altogether, our biochemical analyses support that EPR3 indeed belongs to a functional and unique class of receptors, as shown by the structure. The N-acetyl groups of chitin polymers have previously been demonstrated to be important contact points for LysM proteins\(^{18,21–23}\). Therefore, we investigated if the corresponding O-acetyl groups in EPS are important moieties recognised by EPR3. However, chemical removal of the O-acetyl groups in R7A EPS (deOAc-EPS) did not affect binding \(K_d = 31.3 \pm 11.7 \mu M\), which is similar to that of fully O-acetylated R7A EPS (Fig. 4c). This implies a difference in the ligand perception mechanism between EPR3 and LysM receptors binding chitin ligands, e.g. AICERK1\(^{18}\). In the crystal structure of AICERK1, the position of chitin in the LysM2-binding pocket allows the carbonyl oxygen of the N-acetyl moieties to form hydrogen bonds with backbone amide nitrogens of the main chain\(^{18}\). Such rigorous recognition is unlikely for the O-acetyl groups in EPS as EPS are non-stoichiometrically O-acetylated, in contrast to chitin that has a uniform distribution of N-acetyl groups\(^{10}\). Supporting this notion, we purified and characterised EPS from both R. leguminosarum and S. meliloti with different O-acetylation patterns (Supplementary Fig. 6) and found that these EPS can still be perceived by EPR3. The production of diffusible octasaccharide monomers, corresponding to the main EPS polymer subunits, is not exclusive to R7A, but is present also in R. leguminosarum and S. meliloti, and likely also in other rhizobia, suggesting an important role. Although these rhizobia do not normally infect Lotus, their secreted EPS are detected by EPR3. EPR3 binds R. leguminosarum octasaccharide EPS with a \(K_d = 9.0 \pm 3.7 \mu M\) (Fig. 4d) and S. meliloti EPS (succinoglycan) with a \(K_d = 221.9 \pm 102.3 \mu M\) (Fig. 4e, f), demonstrating that Lotus EPR3 is a promiscuous receptor capable of surveying EPS from different bacterial species while selectively discriminating against...
carbohydrates, such as chitin and maltohexose. One explanation for the stronger EPR3 binding of R. leguminosarum octasaccharide is that R. leguminosarum EPS may be perceived as compatible by Lotus. This explanation is supported by the earlier observation that an R. leguminosarum DZL strain engineered to produce a Nod factor, similar to the Nod factor produced by the nodules, albeit with a delay.

Incompatibility appears to be governed by Nod factor recognition, the first of the two-step recognition mechanism, and not by the second step of EPS recognition. Taken together with the widespread conservation of the EPR3 class of receptors among plants, these results imply that EPR3 and the homologs in non-legume plants are most likely surveillance receptors monitoring carbohydrates from different microbes associated with plant roots. Bacterial EPS is only one group of such carbohydrates. Another example is short-chain and long-chain beta-glucans of bacterial or fungal origin, some of which have been shown to elicit responses in many plants.

In summary, we demonstrate that EPR3 is a defining member of a large and conserved unique class of plant receptors capable of directly perceiving EPS from different bacterial species. This evolutionary conservation highlights a widespread requirement for plants to recognise EPS or other microbial surface carbohydrates, possibly for monitoring associated microbiota. EPR3 contains an intracellular kinase domain predicted to be active. Based on our current knowledge of LysM receptor systems, it is likely that EPR3 is assisted by a co-receptor containing an inactive pseudokinase domain. Future studies in different plant species will help us to better understand this class of receptor and its downstream signalling mechanisms.

**Methods**

**Protein production.** Expression and purification of Lotus japonicus cotype Gifu EPR3 was performed as described previously. In brief, DNA encoding residues 33–232 of EPR3 containing a N-terminal g67 secretion signal and a C-terminal 6xHis-tag was codon-optimised for insect cell expression (GenScript) and inserted into the pOET2 vector (Oxford Expression Technologies). Recombinant baculovirus, used for infecting Sf9 cells cultured in suspension in serum-free HyClone Healthcare, was obtained using the flashBAC GOLD system (OET). Five days post infection, the media was dialysed into buffer containing 50 mM Tris-HCl pH 8.0 and 200 mM NaCl before centrifugation and loaded on a HisTrap excel affinity column (GE Healthcare). The eluted protein was dialysed in buffer containing 50 mM Tris-HCl pH 8.0 and 200 mM NaCl, and further purified on a second HisTrap HP affinity column (GE Healthcare). For crystallisation, EPR3 was treated with PNGase F (1:15 w/w ratio) for 1 h at room temperature and overnight at 4 °C to remove N-linked oligosaccharides. EPR3 was then purified on a Mono S 5/50 column (GE Healthcare) and eluted with a linear gradient of 50–300 mM NaCl and 50 mM Tris-HCl, pH 7.0. Both glycosylated and de-glycosylated EPR3 were purified on a Superdex 75 10/300 column (GE Healthcare) in buffer containing 50 mM KH2PO4 pH 7.8 and 200 mM NaCl for crystallisation and SAXS.

**Nanobody production.** A llama (Lama glama) was immunised four times with 100 μg of purified EPR3. Peripheral blood lymphocytes were isolated from a blood sample, and RNA was extracted using RNase Plus Mini Kit (Qiagen). The total cDNA was generated using the SuperScript III First-Strand Kit (Invitrogen) with random hexamer primers. The coding regions of the nanobodies (Nbs) were amplified by PCR, and inserted into a phagemid vector backbone where the Nbs were C-terminally fused to an E-tag followed by the pIII coat protein. VCSM13 helper phage was used for generating the final M13 phage display Nb library. For selection, EPR3 was biotinylated via primary amine coupling using the Cholamlink NHS labelling system (Solulink) and 20 μg of EPR3 antigen was added to 100 μl of MyOne Streptavidin T1 Dynabeads (Thermo Fisher Scientific) in PBS supplemented with 2% BSA, 15 μg phage particles (2.5 x 1011) were added and incubated with EPR3-coated Dynabeads for 1 h before 15 wash steps with 1 ml of PBS containing 0.1% Tween-20. Phages were eluted by incubating the beads with 0.2 M glycine pH 2.2 for 15 min. The eluted phage particles were amplified and used in a second round of phage display where a reduced amount of EPR3 antigen (2 μg) and fewer M13 phage particles (2.5 x 1010) were used. After two rounds of phage display selections, single colonies were picked and grown in LB medium in a 96-well plate format for 6 h before Nb expression was induced with 0.8 mM IPTG overnight at 30 °C. The 96-well plate was centrifuged, and 50 μl of the supernatant were transferred to an EPR3-coated ELISA plate prepared by coating each well with 0.1 μg of EPR3 and by blocking with PBS containing 0.1% Tween-20 and 2% BSA. After addition of the supernatant, the EPR3-coated ELISA plate was incubated for 1 h, and then washed six times in PBS with 0.1% Tween-20 before anti-E-tag-HPR
antibody (Bethyl) was added at a 1:10,000 dilution. The plate was incubated for 1 h, washed and developed with 3,3′,5,2′-tetramethylbenzidine. The reaction was quenched by the addition of 0.1 M HCl, and the absorbance was measured at 450 nm. Phagosomes from positive cultures were isolated, sequenced and the encoding DNA were cloned into the pET22b(+) (Novagen) for bacterial expression. Nb186 was expressed in E. coli L08STR cells28 that were grown to an optical density of 0.6 at 600 nm before protein expression was induced with 0.2 mM IPTG at 18 °C overnight. Cells were lysed by sonication containing 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM benzamidine and the cleared supernatant was loaded onto a Ni Sepharose 6 FF affinity column (GE Healthcare) and washed prior to elution in lysis buffer supplemented with 500 mM imidazole. Nb186 was finally purified on a Superdex 75 10/300 gel filtration column (GE Healthcare) in buffer containing 50 mM Tris-HCl pH 8.0 and 200 mM NaCl. Complex formation between EPR3-Nb186 was analysed on an analytic Superdex 75 75/300 column (Supplementary Fig. 1a).

Crystalisation and structure determination. Purified de-glycosylated EPR3 and Nb186 were mixed in a 1:1.1 molar ratio and incubated on ice for 1 h before purification on a Superdex 75 10/300 column. The peak fractions containing the EPR3-Nb186 complex were pooled and concentrated on a VivaSpin purifier before being replaced de-glycosylated EPR3 and Nb186 were mixed in a 1:1.1 molar ratio and incubated on ice for 1 h before crystallisation. Crystals were grown at 24 °C in hanging drops containing 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM ethylene glycol and 1 mM benzamidine, and the cleared supernatant was loaded onto a Ni Sepharose 6 FF affinity column (GE Healthcare) and washed prior to elution in lysis buffer supplemented with 500 mM imidazole. Nb186 was finally purified on a Superdex 75 10/300 gel filtration column (GE Healthcare) in buffer containing 50 mM Tris-HCl pH 8.0 and 200 mM NaCl. Complex formation between EPR3-Nb186 complex was built in Coot32 and coordinates and temperature factors were re

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**S. melliloti EPS.** SEC purification of precipitated S. melliloti EPS yielded one major low-molecular mass fraction (LMM EPS). S. melliloti B578 is an ndvB mutant of Rm1021 that is proposed to be deficient in cyclic glucan production while producing normal EPS. The Rm1021 EPS (succinoglycan or EPS-I) is an octasaccharide polymer consisting of seven β-D-Glc and one β-D-Gal residues substituted with 6-O-succinyl, 6-O-acetyl, and 4,6-pyruvyl groups. Composition and glycosyl linkage analysis indicated the presence of 3-substituted Galp, 4-substituted Galp, 6-disubstituted GlcN, 4,6-disubstituted Glcp, 4,6-disubstituted Glcp (likely due to 4,6-substitution with pyruvate), and terminal Glcp. Negative ionisation mode MALDI-TOF-MS analysis demonstrated a heterogeneous mixture of HexHexA, HexA, octasaccharide with a different number of non-cyclic residues, substituents, and major [M−H]− ion at m/z 1565.37, likely due to the fact that octasaccharide was substituted with two O-acetyl and two 4,6-pyruvyl groups. We also detected the structures substituted with hydroxysterbutanoate, but these are not major moieties (Supplementary Fig. 6d).

**MST-binding experiments.** Purified EPR3 was fluorescently labelled using the Monolith NT.115TM Protein Labelling Kit Blue NHS (NanoTemper Technologies) according to the manufacturer’s instructions. All experiments were performed in MST buffer (50 mM K2PO4, pH 7.8, 500 mM NaCl and 0.05% Tween-20) with a constant concentration of EPR3 (100 nM and ~50% labelling efficiency) and dilution series of the various ligands. The samples were incubated for 30 min at room temperature before being loaded into standard capillaries for measurements on a Monolith NT.115 TM instrument (NanoTemper Technologies) at 25 °C, with blue LED power of 50% and MST power of 20%. To accurately measure the experimental errors and ensure data reproducibility, all MST-binding experiments were performed with at least three independently purified samples of EPR3. At the highest ligand concentrations, we occasionally observed weak ligand binding to the fluorescent label itself. To accurately account for this unspecific binding we measured ligand binding to 50 nM free fluorescent label and subtracted this small background contribution to all the respective MST-binding measurements. Binding data were processed with the Prism 7 software (GraphPad Software, Inc.), and the equilibrium-dissociation constant (Kd) values (95% confidence interval) were calculated using the sigmoidal dose–response model.

**SAXS.** A monodisperse peak fraction of EPR3 was collected from a SEC experiment and used for SAXS measurements. Scattering from EPR3 samples without ligand or with R7A EPS (1 mM) added at concentrations ranging from 0.6 to 22.0 mg/ml were collected at the EMBL PETSARA II P12 beamline in a temperature-controlled cell (20 °C) at a wavelength of 1.24 Å. Normalisation and radial averaging were done at the beamline using the automated pipeline. Buffer subtraction and data analysis were performed as previously described37–39. Ab initio low-resolution modelling was performed in DAMMIF (modeling runs) before averaging in DAMAVER and a final refinement step in DAMMIN40. The theoretical scattering profiles of the atomic structures and the experimental data fit were calculated using CRYSTALS. The scattering, Guinier plots and P(r) distance distribution plots were prepared with the Prism 7 software (GraphPad Software, Inc.).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The coordinates and structural factors for the crystal structure of EPR3 has been deposited in the Protein Data Bank under PDB code 6QUP. Source data are provided with this paper.

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J.E.M.M.W.: crystal structure, biochemistry and study design; K.G.: SAXS, biochemistry and study design; T.G.B.: biochemistry; M.V.: protein production; A.M. and P.A.: ligand purification and characterisation; N.S.L.: nanobody production; J.T.S., C.W.R. and A.M.: ligand production; J.S.: study design; K.R.A.: crystal structure, nanobody production and study design. K.R.A. wrote the paper with input from all authors.

Competing interests

J.E.M.M.W., K.G., J.S. and K.R.A. are inventors on a patent application (62888944) submitted by Aarhus University entitled: Modified exopolysaccharide receptors for recognising and structuring microbiota. The remaining authors declare no competing interests.
Additional information

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