An extracellular network of *Arabidopsis* leucine-rich repeat receptor kinases

Elwira Smakowska-Luzan¹*, G. Adam Mott²*, Katarzyna Parys¹*, Martin Stegmann³, Timothy C Howton⁴, Mehdi Layeghifard², Jana Neuhold⁵, Anita Lehner⁵, Jixiang Kong¹, Karin Grünwald¹, Natascha Weinberger¹, Santosh B. Satbhai^{1,6}, Dominik Mayer^{1,7,8}, Wolfgang Busch^{1,6}, Mathias Madalinski^{1,7,8}, Peggy Stolt-Bergner⁵, Nicholas J. Provart^{2,9}, M. Shahid Mukhtar⁴, Cyril Zipfel³, Darrell Desveaux^{2,9}, David S. Guttman^{2,9} & Youssef Belkhadir¹

The cells of multicellular organisms receive extracellular signals using surface receptors. The extracellular domains (ECDs) of cell surface receptors function as interaction platforms, and as regulatory modules of receptor activation^{1,2}. Understanding how interactions between ECDs produce signal-competent receptor complexes is challenging because of their low biochemical tractability^{3,4}. In plants, the discovery of ECD interactions is complicated by the massive expansion of receptor families, which creates tremendous potential for changeover in receptor interactions⁵. The largest of these families in Arabidopsis thaliana consists of 225 evolutionarily related leucine-rich repeat receptor kinases (LRR-RKs)⁵, which function in the sensing of microorganisms, cell expansion, stomata development and stem-cell maintenance⁶⁻⁹. Although the principles that govern LRR-RK signalling activation are emerging^{1,10}, the systems-level organization of this family of proteins is unknown. Here, to address this, we investigated 40,000 potential ECD interactions using a sensitized high-throughput interaction assay³, and produced an LRR-based cell surface interaction network (CSI^{ÎRR}) that consists of 567 interactions. To demonstrate the power of CSI^{LRR} for detecting biologically relevant interactions, we predicted and validated the functions of uncharacterized LRR-RKs in plant growth and immunity. In addition, we show that CSI^{LRR} operates as a unified regulatory network in which the LRR-RKs most crucial for its overall structure are required to prevent the aberrant signalling of receptors that are several network-steps away. Thus, plants have evolved LRR-RK networks to process extracellular signals into carefully balanced responses.

LRR-RKs are modular proteins that feature an ECD with numerous LRR repeats, a transmembrane domain and an intracellular kinase domain¹. LRR-RKs sense a wide array of endogenous and exogenous ligands, including peptides and small molecule hormones, to regulate development and immunity in plants^{7,10}. Stereotypical LRR-RKs include the steroid receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) as well as the immune receptors FLAGELLIN SENSING 2 (FLS2) and PLANT ELICITOR PEPTIDE RECEPTORS 1/2 (PEPR1/2)^{1,11}. Ligand-induced activation of BRI1, FLS2 or PEPR1 and PEPR2 signalling requires physical interaction with the LRR-RK co-receptor BRI1-ASSOCIATED KINASE 1 (BAK1)^{12–15}. In heterotypic LRR-RKs complexes, interactions between ECDs can activate or repress signalling pathways². Yet, the full range of these interactions remains unmapped.

We cloned the ECDs of 200 LRR-RKs from *Arabidopsis* into bait and prey expression vectors for recombinant protein production in Drosophila Schneider S2 cells (Extended Data Fig. 1, Supplementary Table 1). We then implemented the extracellular interaction assay established previously³ and performed an all-by-all screen of the 200 ECDs (Extended Data Fig. 2). Because the Arabidopsis genome encodes 225 LRR-RKs⁵, we interrogated the extracellular LRRs interaction space to a completeness of 79%. This screen resulted in a CSI^{LRR} map containing 2,145 bidirectional interactions, of which only 26.4% (567 high-confidence interactions (HCI)) passed our extremely stringent statistical cut-offs for network construction (Fig. 1a, Supplementary Text 1, Supplementary Table 2). To verify our screen results, the ECDs from the 567 HCI and from a random set of 248 low-confidence interactions (LCI^{CSI}) were independently re-expressed and retested. To benchmark the retest screen, we assembled a positive reference set of 20 literature-curated LRR-RK interaction pairs that complied with the criteria defined previously^{4,16} (Supplementary Table 3). In the retest, the positive reference set, HCI and LCI^{CSI} scored positively at a rate of 100%, 92% and 12.5%, respectively (Extended Data Fig. 3, Supplementary Text 2, Supplementary Table 4). As expected for a high-quality set, the confirmation rates of the HCI and the positive reference set are statistically indistinguishable (P = 0.3894, two-tailed Fisher's exact test).

Models for LRR-RK signalling suggest that ECD interactions help to bring together the intracellular domains (ICDs) for subsequent interaction and signal transduction². We therefore tested whether ICDs from 372 HCI were more likely to interact than another set of 50 randomly selected LRR-RKs via yeast two-hybrid (Y2H) assays (LCI^{Y2H})¹⁷. The HCI and LCI^{Y2H} scored positively at a rate of 54.3% and 10%, respectively (Supplementary Table 5). Notably, of the ICD interactions analysed by Y2H assays, ten were present in our positive reference set, and all tested positively (Supplementary Table 3, Supplementary Text 3). We assign an extremely high level of confidence to interactions that occur at both the ECD and ICD level.

Next, we investigated the biological relevance of CSI^{LRR} interactions by studying the ligand-dependent activation of BRI1- and FLS2mediated signalling¹. We compiled a collection of 27 transfer DNA (T-DNA) insertion mutants¹⁸, targeting the HCI and LCI partners for both BRI1 and FLS2 (HCI^{BRI1/FLS2}/LCI^{BRI1/FLS2}) (Extended Data Figs 4, 5, Supplementary Tables 6, 7). For these T-DNA lines, we used brassinosteroid-induced hypocotyl elongation assays to measure BRI1 activation, and bacterial flagellin peptide (flg22)-induced seedling growth inhibition, peroxidase activity and luminol-based reactive oxygen species (ROS) assays to measure FLS2 activation^{19,20}. Although mutants corresponding to HCI^{BR11/FLS2} partners showed

*These authors contributed equally to this work

¹Gregor Mendel Institute (GMI), Austrian Academy of Sciences, Vienna Biocenter (VBC), Dr Bohr-Gasse 3, 1030 Vienna, Austria. ²Department of Cell & Systems Biology, University of Toronto, 25 Willcocks St., Toronto, Ontario, Canada. ³The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, UK. ⁴Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama, USA. ⁵Protein Technologies Facility, Vienna Biocenter Core Facilities (VBCF), Vienna, Austria. ⁶Salk Institute for Biological Studies, Plant Molecular and Cellular Biology Laboratory, 10010 N Torrey Pines Rd, La Jolla, California 92037, USA. ⁷Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria. ⁸Institute of Molecular Biotechnology GmbH (IMBA), Vienna Biocenter (VBC), Dr. Bohr-Gasse 3, 1030 Vienna, Austria. ⁹Centre for the Analysis of Genome Evolution & Function, 25 Willcocks St., University of Toronto, Toronto, Ontario, Canada.



Figure 1 | **CSI**^{LRR} interaction map and functional validation. a, Interaction heat map organized by phylogenetic subgroups of LRR-RKs (roman numeral, XIV and XV are merged)⁵. The colour scale bar shows interaction score values. b, Hypocotyl length ratios of seedlings grown in the presence or absence of 500 nM brassinolide. See Methods for genotypes and Supplementary Methods for *n*, the number of biologically independent hypocotyls for all genotypes. WT, wild type. $*P = 3.17 \times 10^{-3}$ (*rlk* compared to wild type), $*P = 3.2 \times 10^{-15}$ (all others compared to wild type). NS, not significant. c, flg22-induced seedling growth inhibition (SGI). *n* denotes numbers of biologically independent seedlings. $*P = 3.14 \times 10^{-12}$ (*mik1*); $*P = 2.8 \times 10^{-16}$ (*fls2*), $*P = 2.8 \times 10^{-15}$ (*bak1*), $*P = 2.88 \times 10^{-10}$ (*fir*), $*P = 2.88 \times 10^{-10}$ (*pskr1*). In b and c, wild type (black) and mutant lines targeting the HCI (top interactions; red) and LCI (bottom interactions; yellow) partners for BRI1 and FLS2 are indicated and ordered by decreasing interaction score from left to right. Dots denote

altered signalling outputs (8 out of 8 for BRI1; 3 out of 5 for FLS2), mutants for the LCI^{BR11/FLS2} partners were mostly indistinguishable from wild-type plants (6 out of 7 for BRI1; 7 out of 8 for FLS2) (Fig. 1b, c, Extended Data Figs 4b, 5b–e). Thus, we successfully used CSI^{LRR} to identify functionally relevant interactions for BRI1 and FLS2, and have expanded the repertoire of LRR-RKs known to contribute to plant steroid signalling and flagellin-based

The LRR-RK AT2G27060 (hereafter named FLS2-INTERACTING RECEPTOR, FIR) also interacted with the FLS2 co-receptor BAK1 in CSI^{LRR}, suggesting that FIR may influence the FLS2–BAK1 signal-ling complex *in vivo*. FLS2–BAK1 complex formation was reduced

immunity

individual observations from six independent experiments. Box plots display the first and third quartiles, split by the median; whiskers extend to include the maximum and minimum values. Statistical significance was determined using linear mixed effect modelling, and *P* values are from a post hoc unpaired two-sided *t*-test corrected with the Holm method for multiple testing. **d**, Western blot analyses of FLS2–BAK1 co-immunoprecipitations (co-IP) in seedlings treated with either water (-) or flg22 (+) for 10 min. Anti-BAK1 or anti-FLS2 antibodies were used to analyse lysates from the genotypes indicated. Experiment was repeated three times with similar results. **e**, flg22-induced oxidative bursts represented as total photon counts over 40 min. Genetic backgrounds are indicated. Dots represent individual observations from three independent experiments. Box plots and statistical significance are as in **b** and **c**. *n* denotes numbers of biologically independent leaf discs.

upon flg22 treatment in the *fir* mutant (Fig. 1d), and this correlated with a reduction in flg22-induced ROS burst and *FLG22-INDUCED RECEPTOR KINASE 1* (*FRK1*) gene expression (Fig. 1e, Extended Data Fig. 6a). We also measured flg22-induced root growth inhibition as well as resistance against the bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000), and found that both were significantly reduced in *fir* mutants (Extended Data Fig. 6b–d). Thus, FIR regulates FLS2 signalling and facilitates flg22-induced BAK1–FLS2 complex formation.

Next, we defined the key principles that govern interactions in CSI^{LRR}. LRR-RKs have large (greater than 12 LRR repeats) or small (less than 12 LRR repeats) ECDs, and the sizes are typically associated



Figure 2 | **CSI^{LRR} is defined by four distinct subnetworks and two critical nodes. a**, Expected and observed percentages of interactions organized by interaction types and ECD sizes. The expected percentages were calculated assuming random interaction between observed proteins. **b**, WalkTrap subnetworks are shown in orange. The diameter of the nodes (red circles) is proportional to their PageRank score. Numbers in each node are detailed in Extended Data Fig. 7. Edges (black lines) show interactions between nodes. BAK1 and APEX are marked in black and cyan, respectively. Articulation points are surrounded by a white halo. **c**, Small ECDs have higher PageRank scores than large ECDs. *n* denotes numbers of independent nodes (dots). Statistical significance was determined by an unpaired two-sided *t*-test. Box plots contain the first and third quartiles, split by the median; whiskers extend to include the maximum and minimum values. **d**, Representative rosettes of 20 biologically independent 3-week-old *Arabidopsis* plants. Genetic backgrounds are indicated.

with roles in ligand perception or regulation, respectively^{1,21}. We compared the experimental pattern of interactions between these groups to the expected distribution of interactions assuming random binding (Fig. 2a). The distributions between the subgroups significantly differed from each other (P < 0.0001, chi-square test), indicating that binding events between ECDs in CSI^{LRR} are not random. We observed a four- and tenfold overabundance of homotypic interactions between large and small ECDs, respectively (Fig. 2a), and also detected an increase in heterotypic interactions between small and large ECDs (Fig. 2a). We propose that plants have evolved small LRR-RKs to connect their otherwise unconnected larger counterparts.

Next, we used the WalkTrap algorithm and identified four LRR-RK subnetworks²² (Fig. 2b, Extended Data Fig. 7, Supplementary Table 8), of which at least one is biologically relevant (Supplementary Text 4). The PageRank algorithm was then used to compare the contributions of small and large ECDs to CSI^{LRR} connectivity²³ (Supplementary Table 9). Nodes corresponding to small ECDs have significantly higher PageRank values and are thus more essential to the overall connectivity of the network (Fig. 2c). Notably, BAK1 (a small LRR-RK) was measured by PageRank as the most interconnected and important node in CSI^{LRR}.

Articulation points are nodes whose removal from a network results in the formation of at least two disconnected subnetworks²⁴. Removal of the small LRR-RK AT5G63710 (hereafter named APEX) resulted in the loss of the most nodes from the core structure of CSI^{LRR}, and was thus defined as the most important articulation point for network integrity (Supplementary Table 10). We predicted that genetic elimination of *APEX* and *BAK1* would have obvious developmental consequences. To test this, we constructed *apex bak1-5* doublemutant plants²⁵. Although *apex* and *bak1-5* single-mutant plants were morphologically wild type, *apex bak1-5* double-mutant plants were developmentally impaired (Fig. 2d). Thus, network properties defined *in silico* are relevant in living plants.

In our screen, APEX interacted with PEPR1 and PEPR2. To test whether APEX associates with PEPR1 or PEPR2 in the context of the full-length receptors, we performed co-immunoprecipitation assays. PEPR1 and PEPR2 both associated with APEX in plant cells in the presence or absence of the Pep2 peptide ligand²⁶ (Fig. 3a, b, Supplementary Fig. 1). We next investigated whether the gene dosage of *APEX* would alter PEPR1 or PEPR2 signalling (Extended Data Fig. 8a, b). *apex* knockout plants and two independent overexpression lines (*35S::APEX*) all displayed reduced Pep2-induced bursts of ROS (Fig. 3c). The further reduction in Pep2-triggered ROS bursts in *apex bak1-5* double-mutant plants indicates that both BAK1 and APEX are required for wild-type PEPR1 and PEPR2 signalling (Fig. 3c). Thus, APEX interacts with PEPR1 and PEPR2 in a ligand-independent manner, and a wild-type *APEX* dosage is required for appropriate Pep2-induced responses.

Next, we predicted that changes in APEX dosage would affect the function of CSI^{LRR} as a coherent structural unit *in vivo*, thereby affecting the function of receptors without a direct physical interaction. To test this concept, we analysed whether the functions of BRI1 and FLS2, two receptors that reside several network-steps away from APEX, were affected in our set of APEX lines. The overexpression of APEX had either inconsistent effects or no effects on brassinosteroidinduced hypocotyl elongation and flg22-induced ROS bursts (Fig. 4a, b, Extended Data Fig. 8c). By contrast, BRI1 and FLS2 functions were both altered in apex mutants, as indicated either by the low levels of hypocotyl elongation in response to brassinosteroid or by the enhanced flg22-induced ROS bursts (Fig. 4a, b, Extended Data Fig. 8d, e). Notably, these aberrant ligand-induced signalling responses were both dependent on BAK1 (Fig. 4a, b). Finally, apex mutants showed a notable increase in flg22-induced FLS2-BAK1 complex formation, mitogenactivated protein kinase (MAPK) activation and FRK1 expression (Fig. 4c-e, Supplementary Fig. 1). Thus, elimination of APEX has destabilizing effects in otherwise well-balanced LRR-RK signalling pathways.

To support our contention that the removal of articulation points results in network disruption *in vivo*, we established that mutations in *AT5G51560* (another predicted articulation point in CSI^{LRR}) altered BRI1 function (Extended Data Fig. 9). Our analysis has defined



Figure 3 | APEX interacts with PEPR1 and PEPR2 to regulate danger peptide signalling. a, b, Nicotiana benthamiana leaves expressing Flag-tagged variants of PEPR1 or PERP2 either alone or together with a yellow fluorescent protein (YFP)-tagged APEX were treated with water (-) or Pep2 (+). Western blot analyses of PEPR1-APEX (a) and PEPR2-APEX (b) (co-)immunoprecipitations. Anti-Flag and anti-YFP antibodies were used to analyse lysates. These experiments were repeated three times with similar results. Full scans of the blots are in Supplementary Fig. 1. c, Pep2-induced oxidative bursts represented as total photon counts over 40 min. Genetic backgrounds are indicated. Dots represent individual observations from three independent experiments. n denotes numbers of biologically independent leaf discs. Box plots display the first and third quartiles, split by the median (red line); whiskers extend to include the maximum and minimum values. Statistical significance was determined by linear mixed effect modelling. The letters on top of the boxes (a-e) indicate the results of a post hoc Tukey test; genotypes with the same letter are indistinguishable at >95% confidence.

16 additional LRR-RKs as articulation points of CSI^{LRR} (Supplementary Table 10). Although the removal of any one of these leads to the fragmentation of CSI^{LRR} into no more than three subnetworks, these articulation points make tempting targets to study the LRR-RK family of receptors at the system level.

The mineable resources introduced here have provided insights into the wiring diagram that underlays LRR-RK signalling. We propose that LRR-RKs operate in a unified regulatory network governed by the following key guiding tenets: (i) ligand-induced signalling is modulated locally by the presence and/or activities of other LRR-RKs; (ii) small LRR-RKs, in addition to their function as co-receptors, act as regulatory scaffolds and organize their larger counterparts into a signalling network; and (iii) coupling of LRR-RK signalling to the overall stability of the network ensures appropriate response modulation by network-feedback mechanisms, an overlooked determinant of response specificity.



Figure 4 | CSI^{LRR} functions as a unified regulatory network. a, Hypocotyl length ratios of seedlings grown in the presence or absence of 500 nM brassinolide. Genotypes are indicated (wild type, black). Dots represent individual observations from three independent experiments. n denotes numbers of biologically independent hypocotyls in the presence/absence of brassinolide. b, flg22-induced oxidative burst in leaf discs of the genetic backgrounds indicated. Dots represent individual total photon counts over a 40-min time course; observations are from five independent experiments. n denotes the numbers of biologically independent leaf discs. Box plots in **a** and **b** display the first and third quartiles, split by the median (red line); whiskers extend to include the maximum and minimum values. Statistical significance was determined by linear mixed effect modelling; letters above the boxes (a-c) indicate the results of a post hoc Tukey test. Genotypes with the same letter are indistinguishable at >95% confidence. c, Western blot analyses of FLS2-BAK1 co-immunoprecipitations in seedlings treated with either water (-) or flg22 (+). An anti-BAK1 or anti-FLS2 antibody was used to analyse lysates. Experiment was repeated three times with similar results. d, flg22induced activation of MAPKs in the genotypes indicated. The phosphorylated MPK3/6 proteins were detected with an anti-pERK antibody. Experiment was repeated four times with similar results. Colloidal brilliant blue (CBB) staining shows equal loading of the samples. Full scans of the blots in c and d are presented in Supplementary Fig. 1. e, Seedlings of the genotypes indicated were treated with either water or flg22 and changes in FRK1 transcript levels were quantified by qPCR. Dots represent individual observations from three independent experiments. n = 9 biologically independent mRNA samples for all genotypes. Box plots are as in **a** and **b**. **P* < 0.05, unpaired two-sided *t*-test followed by multiple testing correction using the Holm method.

а

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 13 October 2016; accepted 29 November 2017. Published online 10 January 2018.

- Belkhadir, Y., Yang, L., Hetzel, J., Dangl, J. L. & Chory, J. The growth–defense pivot: crisis management in plants mediated by LRR-RK surface receptors. *Trends Biochem. Sci.* 39, 447–456 (2014).
- Jaillais, Y., Belkhadir, Y., Balsemão-Pires, É., Dangl, J. L. & Chory, J. Extracellular leucine-rich repeats as a platform for receptor/coreceptor complex formation. *Proc. Natl Acad. Sci. USA* **108**, 8503–8507 (2011).
- 3. Özkan, E. *et al.* An extracellular interactome of immunoglobulin and LRR proteins reveals receptor–ligand networks. *Cell* **154**, 228–239 (2013).
- Braun, P. et al. An experimentally derived confidence score for binary protein–protein interactions. Nat. Methods 6, 91–97 (2009).
- Sun, J., Li, L., Wang, P., Zhang, S. & Wu, J. Genome-wide characterization, evolution, and expression analysis of the leucine-rich repeat receptor-like protein kinase (LRR-RLK) gene family in Rosaceae genomes. *BMC Genomics* 18, 763 (2017).
- 6. Shiu, S. H. *et al.* Comparative analysis of the receptor-like kinase family in *Arabidopsis* and rice. *Plant Cell* **16**, 1220–1234 (2004).
- Hohmann, U., Lau, K. & Hothorn, M. The structural basis of ligand perception and signal activation by receptor kinases. *Annu. Rev. Plant Biol.* 68, 109–137 (2017).
- Žipfel, C. & Oldroyd, G. E. Plant signalling in symbiosis and immunity. *Nature* 543, 328–336 (2017).
- Soyars, C. L., James, S. R. & Nimchuk, Z. L. Ready, aim, shoot: stem cell regulation of the shoot apical meristem. *Curr. Opin. Plant Biol.* 29, 163–168 (2016).
- Song, W., Han, Z., Wang, J., Lin, G. & Chai, J. Structural insights into ligand recognition and activation of plant receptor kinases. *Curr. Opin. Struct. Biol.* 43, 18–27 (2017).
- Boutrot, F. & Zipfel, C. Function, discovery, and exploitation of plant pattern recognition receptors for broad-spectrum disease resistance. *Annu. Rev. Phytopathol.* 55, 257–286 (2017).
- Santiago, J., Henzler, C. & Hothorn, M. Molecular mechanism for plant steroid receptor activation by somatic embryogenesis co-receptor kinases. *Science* 341, 889–892 (2013).
- Sun, Y. et al. Structural basis for flg22-induced activation of the Arabidopsis FLS2–BAK1 immune complex. Science 342, 624–628 (2013).
- Sun, Y. et al. Structure reveals that BAK1 as a co-receptor recognizes the BRI1-bound brassinolide. Cell Res. 23, 1326–1329 (2013).
- Tang, J. et al. Structural basis for recognition of an endogenous peptide by the plant receptor kinase PEPR1. Cell Res. 25, 110–120 (2015).
- Braun, P. Interactome mapping for analysis of complex phenotypes: insights from benchmarking binary interaction assays. *Proteomics* 12, 1499–1518 (2012).
- Mukhtar, M. S. *et al.* Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 333, 596–601 (2011).
- Alonso, J. M. et al. Genome-wide insertional mutagenesis of Arabidopsis. thaliana. Science 301, 653–657 (2003).
- Belkhadir, Y. et al. Brassinosteroids modulate the efficiency of plant immune responses to microbe-associated molecular patterns. Proc. Natl Acad. Sci. USA 109, 297–302 (2012).
- Mott, G. A. *et al.* Genomic screens identify a new phytobacterial microbeassociated molecular pattern and the cognate *Arabidopsis* receptor-like kinase that mediates its immune elicitation. *Genome Biol.* **17**, 98 (2016).
- Shinohara, H., Mori, A., Yasue, N., Sumida, K. & Matsubayashi, Y. Identification of three LRR-RKs involved in perception of root meristem growth factor in *Arabidopsis. Proc. Natl Acad. Sci. USA* 113, 3897–3902 (2016).

- Liu, W., Pellegrini, M. & Wang, X. Detecting communities based on network topology. Sci. Rep. 4, 5739 (2014).
- Li, X. Q., Xing, T. & Du, D. Identification of top-ranked proteins within a directional protein interaction network using the PageRank algorithm: applications in humans and plants. *Curr. Issues Mol. Biol.* 20, 13–28 (2016)
- applications in humans and plants. *Curr. Issues Mol. Biol.* 20, 13–28 (2016).
 24. Tian, L., Bashan, A., Shi, D. N. & Liu, Y. Y. Articulation points in complex networks. 8, 14223, doi:10.1038/ncomms14223 (2017).
- Schwessinger, B. *et al.* Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. *PLoS Genet.* 7, e1002046 (2011).

Supplementary Information is available in the online version of the paper.

Acknowledgements We thank A. Pasha for uploading the CSI^{LRR} interaction dataset to the Bio-Analytic Resource for Plant Biology, Y. Dagdas for comments on the manuscript, C. K. Garcia for the pECIA2/14 vectors E. Özkan for his protocols, Y. Saijo, K. Tori and M. Butenko for the pepr1/2, erl2 and hsl2 mutant lines, respectively, and A. Bindeus for help with programming software. This work was supported by grants from the Austrian Academy of Sciences through the Gregor Mendel Institute (Y.B. and W.B.); the Natural Sciences and Engineering Research Council of Canada Discovery Grants to D.S.G. and D.D.; a Canada Research Chair in Plant-Microbe Systems Biology (D.D.) or Comparative Genomics (D.S.G.); and the Centre for the Analysis of Genome Evolution and Function (D.D. and D.S.G.). This research was also funded by the Gatsby Charitable Foundation (C.Z.) and the European Research Council (grant 'PHOSPHinnATE') (C.Z.). E.S.-L. is supported by a Hertha Firnberg Programme post-doctoral fellowship (T-947) from the FWF Austrian Science Fund. M.S. is supported by a post-doctoral fellowship (STE 2448/1) from the Deutsche Forschungsgemeinschaft (DFG). This work was supported by the National Science Foundation (IOS-1557796) to M.S.M. P.S.-B. acknowledges funding from the Austrian Federal Ministry of Science, Research & Economy, and the City of Vienna through the Vienna Biocenter Core Facilities (VBCF). We would like to thank P. Serrano Drozdowskyj, A. Aszodi and A. Gyenesei from the VBCF Biocomputing facility for developing the Platero software. We also thank the VBCF Plant Sciences facilities for the plant growth chambers.

Author Contributions E.S.-L., G.A.M., K.G. and Y.B conceived and designed the experiments for the CSI screen. J.N. and A.L. cloned and expressed all the ECDs with inputs from E.S.-L., P.S.-B. and Y.B.; E.S.-L. performed all the ECD interaction assays. T.C.H. conceived, designed and performed the Y2H assays under the supervision of M.S.M.; G.A.M., E.S.-L. and K.P. characterized and tested all the T-DNA insertion lines under the supervision of D.D., D.S.G. and Y.B.; G.A.M., E.S.-L., K.P., N.W., K.G. and J.K. genotyped and bulked all the T-DNA insertion lines. G.A.M. analysed and implemented the computational and statistical analysis of all the data with inputs from D.S.G. and Y.B.; M.L. and G.A.M. conceived, designed and performed the network analysis with inputs from D.D., D.S.G., N.J.P. and Y.B.; M.S. designed and performed the BAK1-FLS2 co-immunoprecipitation assays under the supervision of C.Z.; K.P. organized and performed the APEX-PEPRs co-immunoprecipitation experiment with guidance from E.S.-L.; E.S.-L. and K.G. generated the apex bak1 doublemutant and 35S::APEX transgenic lines. S.B.S. and W.B. contributed and characterized the *fir* T-DNA mutant. E.S.-L. conceived, organized and performed the physiological assays with brassinosteroids, Pep2 and flg22. D.M. and M.M. devised the synthesis of the flg22 and Pep2 peptides. G.A.M. and Y.B. wrote the manuscript with major input from E.S.-L., M.S.M., C.Z., D.D. and D.S.G.; all authors commented and agreed on the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. Correspondence and requests for materials should be addressed to Y.B. (youssef.belkhadir@gmi.oeaw.ac.at), D.S.G. (david.guttman@utoronto.ca) or D.D. (darrell.desveaux@utoronto.ca).

METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

Molecular cloning of LRR-RK extracellular domains. For each ECD cloned, we determined the boundaries of signal peptides and transmembrane domains using a range of bioinformatics tools²⁷. A key step in defining the boundaries of each ECD was the identification of the N- and C-terminal cysteine-capping consensus motifs (CXXXXC and variations thereof) that border most of the Arabidopsis ECDs. This was achieved by visual inspection of the primary amino acid sequences. These cysteine caps are thought to cap the exposed edges of the hydrophobic core formed by the repetition of the LRRs and produce disulfide bonds that preserve the tertiary protein structure. We found that the cysteine caps were important for enhancing ECD solubility and preventing aggregation and proteolysis in vitro. For expression in Drosophila melanogaster Schneider 2 (S2) cells, each ECD was inserted into the pECIA-2 and the pECIA-14 expression vectors (a gift from C. K. Garcia)³. pECIA2/14 are derivatives of the pMT/BiP/V5 (Invitrogen, V4130-20), which uses a copper-inducible Drosophila metallothionein promoter and have the signal sequence of the Drosophila BiP protein. The ECDs were cloned by sequence and ligation independent cloning (SLIC) between the existing BiP signal sequence and the C-terminal epitope tags specific to each vector. Sanger sequencing confirmed the presence of each insert. Primers were designed to have a sequence partially homologous to the desired boundaries of the ECDs followed by extensions for RecA-mediated SLIC strategy attached (Supplementary Table 1). Amplification was done using Phusion Flash Mastermix Thermo Fisher Scientific according to the manufacturer's instructions for 2-step PCR. ECDs (176 out of 200) were cloned from plasmid templates available from the Arabidopsis Biological Resource Center (ABRC)²⁸. Twenty-four ECDs were cloned from Arabidopsis seedlings and mature leaves using RT-PCR, followed by amplification as described above and by RecAmediated SLIC.

Secreted expression of LRR-RK extracellular domains. The ECDs cloned into the pECIA2 (for expression as a bait) and pECIA14 (for expression as a prey) vectors were expressed using transient transfection of *Drosophila* S2 cells cultured at 27 °C. Upon transfection using Effectene (Qiagen), the culturing temperature was changed to 21 °C. Twenty-four hours after transfection, protein expression was induced with 1 mM CuSO₄ and supernatant was collected three days after induction. Protease inhibitors (Sigma) and 0.02% NaN₃ were added to the medium (ESF 921, Expression Systems) containing the recombinant ECDs and then stored at 4 °C before use. The cell supernatant was assessed for recombinant protein expression by western blotting using anti-V5 antibodies (Invitrogen) for the baits or by alkaline phosphatase activity quantification for the preys.

CSI^{LRR} primary screen. Pairwise interaction assays were performed as detailed previously³ for the extracellular interactome assay (ECIA) with the slight modifications indicated below. Schneider's medium containing recombinant ECDs was subjected to a fourfold dilution in a PBS buffer containing 1 mM CaC12, 1 mM MgCl₂ (equilibration buffer) and 0.1% bovine serum albumin (BSA; Sigma). Bait proteins fused to the Fc domain were captured directly on 96-well protein-A-coated plates (Thermo Fisher Scientific) by overnight incubation at 4°C. Protein-A-coated plates were washed in a PBS solution containing 0.1% Tween-20 before use. The bait-coated plates were blocked with the equilibration buffer containing 1% BSA for 3 h at 4 °C and subsequently washed. The prey proteins fused to the alkaline phosphatase were then added to the wells and incubated for 2h at 4°C and then washed away before adding the alkaline phosphatase substrate (KPL 50-88-02). Upon addition of the substrate, plates were incubated for 2 h at room temperature and alkaline phosphatase activity was monitored by measuring the absorbance at 650 nm using a Synergy H4 Multi-Mode plate reader (BioTek). Images of the 96-well plates were acquired for visual inspection. The complete set of raw absorbance values was combined into a binary dataset using an in-house-designed script (Platero v0.1.4), and then subjected to post experimental statistical analysis to remove both false positive and false negative interactions.

CSI^{LRR} data analysis. The complete set of absorbance values for each pairwise interaction was combined into a data matrix. To make measurements comparable across plates and eliminate any bias in the data arising from the differential background binding capacities of the baits and preys we used a two-way median polish^{29,30}. The residuals were then used to calculate the median and median absolute deviation (MAD). The MAD is the median of the absolute values of the residuals (deviations) from the data's median. The MAD was used for the calculation of modified *Z*-scores for each individual interaction measured. The modified *Z*-score used here is (i) nonparametric and makes minimal distributional assumptions, (ii) minimizes measurement bias due to positional effects and (iii) is resistant to statistical outliers. The modified *Z*-score usually excludes control measurements altogether under the assumption that most interactions in a screen such as CSI^{LRR}

would be unproductive and thus serve as controls. However, during the primary screen each 96-well plate contained two mock prey negative control wells and one well with the positive control interaction pair BAK1–BIR4³¹. To identify high-stringency bidirectional interactions, we calculated the geometric mean modified *Z*-score of the interaction as measured in the bait–prey and prey–bait orientations. Any value for which the geometric mean product of the *Z*-scores was greater than 2.5 was considered significant for the purposes of network construction.

CSI^{LRR} retest screens. All of the HCI in CSI^{LRR} and a randomly selected subset of LCI^{CSI} were independently retested. Each ECD was newly expressed and all retested interactions were assayed in both bait-prey orientations. For each interaction tested, three prey-only negative control wells were included, to control for non-specific binding. Thus, a total of six negative controls were tested for each bidirectional interaction. One well containing the positive control interaction pair BAK1-BIR4 was included on each plate. The two-way median polish and modified Z-scoring system used in the initial screen depends upon large numbers of non-interactions to perform reliably. The low sample number, enriched with high or low performing protein pairs, led to an asymmetrical data distribution in the retest, making it inappropriate to implement our original hit calling method. Instead, we implemented a multi-stage hit calling process to ensure reliable data confirmation. The absorbance values were paired with the corresponding value from the CSI^{LRR} and subjected to an interquartile range normalization step to ensure the two datasets could be accurately compared (Extended Data Fig. 3). The geometric mean of the normalized absorbance values for each bidirectional interaction was then calculated. The threshold for inclusion in the positive interaction set was set to the lowest geometric mean absorbance value found in the 567 interactions present in the CSI^{LRR} (absorbance value = 0.090989). Therefore, any interaction with a geometric mean absorbance value > 0.090989 was considered positive; all others were considered negative.

CSI^{LRR} network construction and analysis. The network was constructed using the igraph package (http://igraph.org/r/) in the R programming environment (https://www.r-project.org/). To identify clusters of interacting proteins in the network, we used the WalkTrap algorithm as implemented in igraph; this algorithm is based on the concept that if one performs random walks on a network, then the walks are more likely to stay within the densely connected parts of the network, thus corresponding to clusters with higher levels of interconnectedness²². The WalkTrap was implemented with edge weights corresponding to the interaction score and a length of random walk of 8. To measure the importance of each node within the network, we applied the PageRank algorithm as implemented in igraph, which operates by counting the number and quality of links to a node, thereby establishing its importance and assigning a 'weight' value to it²³. In simpler terms, PageRank measures node connectivity via the number of connections to other nodes. The PageRank algorithm is an example of a link-analysis algorithm, which are iterative and interactive data analysis techniques that operate with the underlying assumption that nodes with higher scores are likely to be more connected to other nodes when compared to nodes with lower scores²³. The PageRank implementation using the PRPACK library within the igraph package was used with edge weights corresponding to the interaction score, and a damping factor of 0.85, which is also the default. Finally, we identified the articulation points (or cut vertices) in the network. An articulation point is any node in a unidirectional network the removal of which disconnects the network.

Y2H assays with LRR-RK ICDs. The Y2H experiment was conducted as described previously17 with some modifications. In brief, we used a collection of LRR-RK ICDs cloned in both bait and prey plasmids¹⁷. The ICDs of the LRR-RKs were fused to the GAL4 activation domain using a pDEST-AD-CHY2 vector with a tryptophan selection marker to form the prey constructs and to the GAL4 DNA-binding domain using a pDEST-DB vector with a leucine selection marker to form the bait constructs. Target prey and bait constructs were transformed into Saccharomyces cerevisiae strains Y8800 (MATa) and Y8930 (MATa), respectively. Transformations were confirmed by selecting the haploid yeast strains on their corresponding selective medium (SD-T and SD-L). Haploid bait and prey strains were mated in liquid YEPD (yeast extract 10 g l⁻¹, peptone 20 g l⁻¹, dextrose 20 g l⁻¹, adenine 100 mg l⁻¹) medium overnight at 30 °C. The resulting diploid yeasts were selected in liquid SD-LT medium for 48h at 30 °C. Reconstitution of the GAL4 transcription factor through the interaction of the bait and prey led to the activation of a HIS3 reporter gene and subsequently biosynthesis of histidine. Because the pDEST-AD vector contains the CHY2 (a cycloheximide-sensitive gene), any growth on the yeast medium containing cycloheximide constitutes a false positive interaction. Equal amounts of diploid yeasts were transferred to solid SD-LTH (positive selection plates) and SD-LH+ cycloheximide (20 mg l⁻¹) medium (de novo autoactivation plates). Interactions were scored positive if there was growth on positive selection plates, but no growth on *de novo* auto-activation plates. The retest on the random $LCI^{Y_{2H}}$ pairs was performed in similar conditions.

T-DNA insertions of top and bottom BRI1- and FLS2-interaction partners. Noting that our statistical cut-off for considering an interaction for network construction was set to a CSI score (geometric mean modified Z-score) > 2.5, we compiled a list of 'top-interactions' (HCI^{BRI1/FLS2}; CSI score > 1.75) and 'bottom-interactions' (LCI^{BRI1/FLS2}; CSI score = 0) (Supplementary Table 6). We amassed a collection of T-DNA insertion lines from the *Arabidopsis* Biological Resource Centre (ABRC) for the HCI^{BR11/FLS2} and LCI^{BR11/FLS2} genes, focusing when possible on exon insertions closest to the 5' end of each gene. For each of the mutant lines we performed PCR tests for the presence of non-segregating (homozygous) T-DNA insertions in each target gene as well as qPCR analysis of altered target gene expression (Extended Data Figs 4, 5, Supplementary Table 7). For BRI1, we tested mutant lines targeting the following interaction partners: HCI^{BRI1} top genes; first rank: STRUBBELIG-RECEPTOR FAMILY 9 (SRF9)³², second rank: ERECTA-LIKE 2 (ERL2)³³, third rank: FIR/AT2G27060 (this study), fourth rank: BAK1 (bak1-4 allele)^{25,34,35}, sixth rank: BARELY ANY MERISTEM 3 (BAM3)³⁶, seventh rank: SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 4 (SERK4)^{35,37}, eighth rank: RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1)³⁸ and ninth rank: HAESA-LIKE 2 (HSL2)³⁹⁻⁴¹. We were not able to test the following genes; fifth rank: RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2)⁴² and tenth rank: BAK1 INTERACTING RECEPTOR 4 (BIR4)³¹. The mutant lines obtained from the Salk Institute (La Jolla; the SALK lines collection, http://signal.salk.edu/cgibin/tdnaexpress) were annotated as homozygous for the T-DNA inserts but we genotyped both as wild-type plants. LCIBRII bottom genes; 191st rank: RECEPTOR-LIKE KINASE (RLK)43, 193rd rank: REDUCED IN LATERAL GROWTH1 (RUL1)⁴⁴, 194th rank: SENESCENCE-ASSOCIATED RECEPTOR-LIKE KINASE (SARK)⁴⁵, 196th rank: STERILITY REGULATING KINASE MEMBER 1 (SKM1)⁴⁶, 197th rank: SUPPRESSOR OF BIR1-1 (SOBIR1)⁴⁷, 198th rank: STRUBBELIG-RECEPTOR FAMILY 4 (SRF4)³² and 200th rank: TRANSMEMBRANE KINASE LIKE 1 (TMKL1)⁴⁸. The following genes were not tested; 192th rank: RECEPTOR-LIKE KINASE 902 (RLK902)⁴⁹ and 199th rank: TRANSMEMBRANE KINASE 1 (TMK1)⁵⁰. Although annotated as homozygous for the T-DNA insert in the SALK database, we genotyped both lines as wild-type plants.

For FLS2, we tested mutant lines targeting the following HCI^{FLS2} top genes; first rank: MDIS1-INTERACTING RECEPTOR LIKE KINASE 1 (MIK1)⁵¹, second rank: *FLS2* as an internal control but also as a self-interaction⁵², third rank: FIR/AT2G27060 (this study), fifth rank: BAK1 (bak1-4 allele)^{25,34,35}, seventh rank: AT5G62710, eighth rank: BARELY ANY MERISTEM 3 (BAM3)³⁶, 13th rank: RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1)38, 14th rank: STRUBBELIG-RECEPTOR FAMILY 9 (SRF9)³² and 15th rank: AT2G27060. We did not test the following; fourth rank: ERECTA and sixth rank: ERECTA-LIKE 2 (ERL2) because the er mutant shows altered flg22-induced marker gene expression³³, and tenth rank: IMPAIRED-OOMYCETE SUSCEPTIBILITY 1 (IOS1), which has been implicated in flg22-induced ROS burst, marker gene expression and FLS2-BAK1 complex formation⁵³. The following T-DNA lines were not tested; fifth rank: RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2)42, eleventh rank: RECEPTOR-LIKE KINASE 1 (RKL1)⁴⁹ and twelfth rank: BAK1 INTERACTING RECEPTOR 4 $(BIR4)^{31}$ because we genotyped them as wild type despite their annotation as homozygous for the presence of a T-DNA insert. $\rm LCI^{FLS2}$ bottom genes; 190th rank: phytosulfokine peptide receptor 1 (PSKR1)54-56, 191st rank: PEPR257, 192nd rank: AT3G46350, 194th rank: AT3G14840, 195th rank: AT2G01210, 196th rank: PEPR1⁵⁷, 198th rank: FEI2³¹ and 200th rank: NSP-INTERACTING KINASE 3 (NIK3)58.

Brassinosteroid hypocotyl responses assays. These assays have been performed as described previously^{2,19}.

Peroxidase flg22 responses assays. The peroxidase assay was carried out as described previously²⁰. In brief, leaf discs were taken from 4-week-old *A. thaliana* plants. The discs were washed for 1 h in 1 ml of 1 × MS solution with agitation. After washing, discs were transferred to individual wells of a clear 96-well assay plate avoiding the use of the edge wells to minimize evaporation effects. Each well received 50 µl of 1 × MS buffer alone, or supplemented with 1 µM of flg22 peptide. Plates were sealed with parafilm and incubated for 20 h with agitation. The leaf discs were removed and each well received 50µl of a 1 mg ml⁻¹ solution of 5-aminosalicylic acid (A79809, Sigma-Aldrich) pH 6.0 with 0.01% hydrogen peroxide. The reaction proceeded for 1–3 min and was stopped by the addition of 20µl 2 N NaOH before reading the OD_{600 nm} on a POLARstar OPTIMA microplate reader (BMG Labtech). The flg22 peptide was obtained from Genscript.

Transient expression in *Nicotiana benthamiana.* Agrobacterium tumefaciens GV3101 strains were grown in LB medium supplemented with appropriate antibiotics overnight. Cultures were spun down and re-suspended in 10 mM MgCl₂ to $OD_{600 \text{ nm}} = 0.1$. Agrobacterium strains carrying the pB35GWF binary plant expression vector for the expression of the full-length coding regions of PEPR1 (S1G73080BFF)²⁸ and PEPR2 (S1G17750BFF)²⁸ fused to a C-terminal Flag epitope tag were constructed and used for immunoprecipitation and western blot

assays. pDONR-Zeo vector (Life Technologies) containing the cDNA of *APEX* (N5G63710ZEF) was used for gateway recombination in the binary plant expression vector pEarleyGate101 vector to generate the C-terminal YFP–HA tag fusion vector expressing *APEX-YFP-HA* under the control of the CaMV35S promoter. For each of the protein interaction pairs tested, the respective sets of agrobacterium strains were mixed 1:1 and syringe infiltrated into 3-week-old *N. benthamiana* leaves of plants grown in short-day conditions (12 h light:12 h dark). Samples for protein extraction were collected three days after infiltration before flash-freezing in liquid nitrogen.

Protein extraction and immunoprecipitation in *N. benthamiana.* Leaves were ground in liquid nitrogen and extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM DTT, 10 mM EDTA, 1 mM NaF, 1 mM Na₂MoO₄•2H₂O, 1× (v/v) cOmplete Tablets, EDTA-free (Roche), and 1% (v/v) IGEPAL CA-630 (Sigma-Aldrich)) was added at 2 ml per gram tissue powder. Samples were homogenized by alternate rounds of Polytron and incubated in extraction buffer for 1 h at 4°C. Samples were the clarified by a 20-min centrifugation step at 4°C and 16,000g. Supernatants (3 ml) were adjusted to 2 mg ml⁻¹ protein and incubated for 3 h at 4°C with 30µl GFP Trap-A beads (Chromotek) with slow but constant rotation. Following incubation, beads were washed four times with washing buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% PMSF, and 0.1% IPEGAL. One hundred microlitres of 5× SDS Laemmli buffer was added to the beads, and the beads were heated at 95°C for 10 min and subjected for further SDS–PAGE and immunoblotting analysis.

Plant cultivation, transgenic plants and mutants. The wild type used in all experiments was A. thaliana accession Columbia (Col-0). Unless specified otherwise, the apex-1 allele was used in this work (Extended Data Fig. 8a). Plants were grown on soil or vertically on Petri dishes containing 0.5× Murashige and Skoog medium in long-day light conditions (16h light:8h dark). For Pto DC3000 pathogen assay and callose deposition upon flg22 treatment, plants were grown in short-day conditions (12h light:12h dark). The mutant plant genotypes used in this work are listed in Supplementary Table 7. For overexpression studies, the 35S::APEX-YFP-HA construct was transformed separately into wild-type plants and more than 20 independent T1 lines were isolated and between three and eight representative mono-insertion lines were selected in the T2 generation. DNA genotyping, epifluorescence microscopy and protein extraction were performed on segregating T₂ to obtain homozygous T₃ generation lines with maximal expression levels (Extended Data Fig. 8b). The double-mutant apex-1 bak1-5 was generated by crosses and genotyped for homozygosity using allele-specific primers for apex-1 and dCAPS marker for bak1-5 as described previously25. Genotyping was repeated for two consecutive generations and confirmed by Sanger sequencing. Primers are listed in Supplementary Table 7.

Protein extraction and immunoprecipitation in Arabidopsis. Fifteen to twenty seedlings were grown in each well of a 6-well plate for 2 weeks. Subsequently, seedlings were transferred to water and incubated overnight. The next day, flg22 was added at a final concentration of 100 nM and incubated for 10 min. Seedlings were than frozen in liquid nitrogen and subjected to protein isolation. To analyse FLS2-BAK1 receptor complex formation, proteins were isolated in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM dithiothreitol, 1% protease inhibitor cocktail (Sigma-Aldrich), 2 mM Na2MOO4, 2.5 mM NaF, 1.5 mM activated Na3VO4, 1 mM phenylmethanesulfonyl fluoride and 1% IGEPAL. For immunoprecipitations, anti-rabbit Trueblot agarose beads (eBioscience) coupled with anti-FLS2 antibodies and incubated with the crude extract for 2-3h at 4°C. Subsequently, beads were washed three times with wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride and 0.5% IGEPAL) before adding Laemmli sample buffer and incubating for 10 min at 95 °C. Analysis was carried out by SDS-PAGE and western blots using anti-FLS2 and anti-BAK1 antibodies²⁵. Protein analysis. In all our protein manipulations, equal loading was ensured by Bradford protein quantification before loading and by CBB or Red Ponceau staining of the membrane post-protein transfer. Anti-GFP-HRP (MACS) and anti-Flag-HRP (Sigma-Aldrich) antibodies were used according to manufacturer's instructions. Polyclonal anti-FLS2 and anti-BAK1 antibodies were used as described previously²⁵. Signal detection was achieved through chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific) and detected using autoradiography films (CL-XPosure Film, Thermo Fisher Scientific).

RNA isolation, cDNA synthesis and qPCR analysis. Total RNA was isolated from 1-week-old seedlings grown on 1/2 MS plates using either the GeneMATRIX Universal RNA Purification Kit (EURx) or TRI Reagent (Sigma-Aldrich), followed by DNaseI treatment (Thermo Fisher Scientific). Reverse transcription reactions were performed using up to 2 μ g of total RNA and a reverse transcription kit (Applied Biosystems or Life Technologies). The cDNAs were used as a template for qPCR. qPCR analyses were performed using a Roche LightCycler96 instrument (Roche Applied Science) and data were analysed using the LightCycler 96 version

1.1 software or BioRad C1000 thermal cycler (BioRad). FastStart Essential DNA Green Master (Roche) or Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) were used according to manufacturer's instructions. Material from wild type plant served as the calibrator, and *ACTIN* or *UBQ10* was used as a reference. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_{t}}$ method. The amplification protocol consisted of: 95 °C for 1 min, (95 °C for 10 s, 55–62 °C for 10 s, 72 °C for 20 s) × 44 cycles. The relative mRNA levels were determined by normalizing the PCR threshold cycle number with *ACTIN* or *UBQ10*. All experiments were repeated three times independently, and the mean was calculated. The specificity of the amplification products was verified by melting curve analysis.

MAMP and DAMP responses assays. flg22 (QRLSTGSRINSAKDDAAGLQIA) and pep2 (DNKAKSKKRDKEKPSSGRPGQTNSVPNAAIQVYKED) peptides were synthesized at >95% purity by the in-house protein chemistry facility and dissolved to a 10 mM stock in pure water. For ROS burst assays, leaf disks (diameter 6 mm) were cut out from 4-5-week-old plants. Single disks were placed adaxial side up into 96-well microtitre plates in which every well contained 200 µl sterile MonoQ water. Floated disks were then vacuum infiltrated for 10 min. The plates were incubated on a rocking table at 45 r.p.m. in continuous light, at 21 °C for 5 h. The mix for elicitation containing 9.91 ml of MonoQ water, $40 \mu l$ of $500 \times$ HRP, $40 \mu l$ of $500 \times L-012$ and appropriate peptide at a final concentration of $1 \mu M$ was freshly prepared in falcon tubes wrapped with aluminium foil on ice. For each well the water was carefully removed and replaced immediately with 100 µl of elicitation mix using a multichannel pipette. Relative luminescence measurements were started immediately after adding the elicitation mix using a BiTec Synergy 4 microplate reader. Horseradish peroxidase (HRP) was purchased from Sigma-Aldrich and prepared at a 10 mg ml^{-1} (500×) concentration in sterile MonoQ water. L-012 was purchased from Wako Chemicals GmbH. Preparation of a $500 \times$ L-012 stock solution containing 17 mg ml⁻¹ L-012 in sterile MonoQ water and was subsequently protected from light. Solutions were stored at -20 °C. For the analysis of ROS burst data, the models were constructed using the total relative light units measured for the first 39 time points to ensure comparability across experiments. Root inhibition ratios were calculated on 7-day-old seedlings grown on plates left untreated or treated with 1 µM flg22.

Seedling growth inhibition assay. Seedlings of the noted *A. thaliana* lines were grown for 5 days on MS-agar plates with 1% sucrose before transfer of up to 10 seedlings to each well of a 6-well plate containing 1 ml of $0.5 \times$ MS medium with 1% sucrose. The seedlings were treated with water or 100 nM flg22 peptide and grown further for 7 days. The seedlings were removed, briefly dried, and weighed (fresh weight). The percentage of seedling growth inhibition was calculated by dividing the weight of individual treated seedlings by the mean weight of the control non-treated seedlings of the same genotype. The percentage of seedling growth inhibition was calculated by dividing the weight of 10 non-treated seedlings of the same genotype. A maximum of 10 seedlings of each genotype were treated and the experiment was performed six times.

Pathogens assays. Assays with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) have been previously described¹⁹. Bacterial growth in plant leaves was assessed by inoculating 4-week-old plants with a bacterial inoculum of 10^5 colony-forming units (cfu) ml⁻¹. Growth inhibition of *Pto* DC3000 by 1 μ M flg22 was conducted as described¹⁹. Leaves were either infiltrated with water or with an elicitor solution containing 1 μ M flg22. For each sample, four leaf discs were pooled and three samples were taken per data point (12 leaf discs in total). Leaf discs were bored from the infiltrated area and ground to homogeneity in 10 mM MgCl₂. The bacterial titre was determined by plating and serial dilution.

Program used for modelling and statistical analysis. Statistical analysis was performed using linear mixed effect modelling in the R programming environment (https://www.r-project.org/). Before modelling, data from independent experiments were combined and outliers were removed using the ROUT method, as implemented in GraphPad PRISM 7.0 (Q = 0.1%) (GraphPad Software, http:// www.graphpad.com). Each dataset was checked for normality to ensure accurate modelling. qPCR data were analysed as fold induction, whereas all other data were log₁₀ transformed before modelling to improve fit. Linear mixed effect models were constructed using the lme4 package: https://cran.r-project.org/package=lme4, using the genotype as a fixed effect and the individual experiment as a random effect. The resulting models were inspected for fit and further outlier checks were accomplished by examining both the Cook's distance and dfbeta distributions using the LMER Convenience Functions and influence.ME packages (https://CRAN.Rproject.org/package=LMERConvenienceFunctions; https://cran.r-project. org/web/packages/influence.ME/). Statistical significance was determined using the lmerTest package (https://cran.r-project.org/package=lmerTest) using the Satterthwaite approximation and the resulting P values were corrected for multiple testing using the Holm method. In cases where pairwise comparisons were

required, the adjusted *P* values were calculated using Tukey's honest significant difference (HSD) as implemented in the multcomp package (https://cran.r-project. org/package=multcomp).

To calculate the expected binding frequencies of a random network, we classified each node based on its ECD. Assuming equal frequency of a given node binding to any other node, the frequency for each class of binding event was calculated and divided into self-interactions between small ECDs (small–small homotypic), self-interactions between large ECDs (large–large homotypic), interactions between two different small ECDs (small heterotypic), interactions between two different large ECDs (large heterotypic), and interactions between one small and one large ECD (small–large heterotypic).

To estimate the reliability of the estimates provided by the retest screen (Extended Data Fig. 3d), the observed rate of interactions found in the CSI and retest sets were used for a Monte Carlo simulation. Sets of observations were selected at random from these populations, with the number of observations equal to the number present in the retest sets. This process was completed 100,000 times. These values were used to calculate the mean and s.d. of the samplings.

Details about the linear mixed effect modelling can be found in the Supplementary Methods.

Data and software accessibility. The data supporting the findings of this study are available within the paper and its Supplementary Information files. Source data for Figs 1, 2, 3, 4, Extended Data Fig. 2, 3, 4, 5, 6, 8 and 9 are provided with the paper. All the raw absorbance reads related to the ECD interaction screen are available in the Supplementary Table 11. The high-confidence LRR-RKs interaction dataset is publically available online at the Bio-Analytic Resource under accession (MI 2189, Smakowska-Luzan et al. 2018, doi:10.1038/nature25184): http://bar.utoronto.ca/ interactions. The custom PLATERO script used for concatenating the interaction absorbance values is available upon request from the corresponding author or from https://github.com/AdamMott/platero-code.

- Couto, D. & Zipfel, C. Regulation of pattern recognition receptor signalling in plants. Nat. Rev. Immunol. 16, 537–552 (2016).
- McWilliam, H. et al. Analysis Tool Web Services from the EMBL-EBI. Nucleic Acids Res. 41, W597–W600 (2013).
- Gou, X. et al. Genome-wide cloning and sequence analysis of leucine-rich repeat receptor-like protein kinase genes in Arabidopsis thaliana. BMC Genomics 11, 19 (2010).
- Malo, N., Hanley, J. A., Cerquozzi, S., Pelletier, J. & Nadon, R. Statistical practice in high-throughput screening data analysis. *Nat. Biotechnol.* 24, 167–175 (2006).
- Brideau, C., Gunter, B., Pikounis, B. & Liaw, A. Improved statistical methods for hit selection in high-throughput screening. J. Biomol. Screen. 8, 634–647 (2003).
- 31. Halter, T. et al. The leucine-rich repeat receptor kinase BIR2 is a negative regulator of BAK1 in plant immunity. *Curr. Biol.* **24**, 134–143 (2014).
- Eyüboglu, B. et al. Molecular characterisation of the STRUBBELIG-RECEPTOR FAMILY of genes encoding putative leucine-rich repeat receptor-like kinases in Arabidopsis thaliana. BMC Plant Biol. 7, 16 (2007).
- Jordá, L. et al. ERECTA and BAK1 receptor like kinases interact to regulate immune responses in Arabidopsis. Front. Plant Sci. 7, 897 (2016).
- Russinova, E. et al. Heterodimerization and endocytosis of Arabidopsis brassinosteroid receptors BRI1 and AtSERK3 (BAK1). Plant Cell 16, 3216–3229 (2004).
- Roux, M. et al. The Arabidopsis leucine-rich repeat receptor-like kinases BAK1/ SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell* 23, 2440–2455 (2011).
- Hazak, O. *et al.* Perception of root-active CLE peptides requires CORYNE function in the phloem vasculature. *EMBO Rep.* 18, 1367–1381 (2017).
- Meng, X. et al. Differential function of Arabidopsis SERK family receptor-like kinases in stomatal patterning. Curr. Biol. 25, 2361–2372 (2015).
- Nodine, M. D., Yadegari, R. & Tax, F. E. RPK1 and TOAD2 are two receptor-like kinases redundantly required for *Arabidopsis* embryonic pattern formation. *Dev. Cell* **12**, 943–956 (2007).
- Wang, X. et al. IDL6-HAÈ/HSL2 impacts pectin degradation and resistance to Pseudomonas syringae pv tomato DC3000 in Arabidopsis leaves. Plant J. 89, 250–263 (2017).
- Meng, X. et al. Ligand-induced receptor-like kinase complex regulates floral organ abscission in Arabidopsis. Cell Reports 14, 1330–1338 (2016).
- 41. Santiago, J. *et al.* Mechanistic insight into a peptide hormone signaling complex mediating floral organ abscission. *Elife* **5**, e15075 (2016).
- Nimchuk, Z. L. CLAVATA1 controls distinct signaling outputs that buffer shoot stem cell proliferation through a two-step transcriptional compensation loop. *PLoS Genet.* 13, e1006681 (2017).
- Cole, S. J. & Diener, A. C. Diversity in receptor-like kinase genes is a major determinant of quantitative resistance to *Fusarium oxysporum* f.sp. *matthioli*. *New Phytol.* **200**, 172–184 (2013).
- Agusti, J., Lichtenberger, R., Schwarz, M., Nehlin, L. & Greb, T. Characterization of transcriptome remodeling during cambium formation identifies MOL1 and RUL1 as opposing regulators of secondary growth. *PLoS Genet.* 7, e1001312 (2011).



- 45. Xiao, D. et al. SENESCENCE-SUPPRESSED PROTEIN PHOSPHATASE directly interacts with the cytoplasmic domain of SENESCENCE-ASSOCIATED RECEPTOR-LIKE KINASE and negatively regulates leaf senescence in Arabidopsis. Plant Physiol. **169**, 1275–1291 (2015). Kang, Y. H. & Hardtke, C. S. Arabidopsis MAKR5 is a positive effector of
- 46.
- Adlig, f. n. & nardike, c. S. Arabidopsis MANG is a positive electron of BAM3-dependent CLE45 signaling. *EMBO Rep.* **17**, 1145–1154 (2016). Albert, I. *et al.* An RLP23–SOBIR1–BAK1 complex mediates NLP-triggered immunity. *Nat. Plants* **1**, 15140 (2015). 47 48
- Valon, C., Smalle, J., Goodman, H. M. & Giraudat, J. Characterization of an Arabidopsis thaliana gene (TMKL1) encoding a putative transmembrane protein with an unusual kinase-like domain. Plant Mol. Biol. 23, 415–421 (1993).
- 49. Tarutani, Y. et al. Molecular characterization of two highly homologous receptor-like kinase genes, RLK902 and RKL1, in Arabidopsis thaliana. Biosci. Biotechnol. Biochem. 68, 1935-1941 (2004).
- 50. Chang, C. et al. The TMK1 gene from Arabidopsis codes for a protein with structural and biochemical characteristics of a receptor protein kinase. Plant Cell 4, 1263-1271 (1992).
- 51. Wang, T. et al. A receptor heteromer mediates the male perception of female attractants in plants. Nature 531, 241-244 (2016).

- 52. Sun, W. et al. Probing the Arabidopsis flagellin receptor: FLS2-FLS2 association and the contributions of specific domains to signaling function. Plant Cell 24, 1096-1113 (2012).
- 53 Yeh, Y. H. & Panzeri, D. The Arabidopsis malectin-like/LRR-RLK IOS1 is critical for BAK1-dependent and BAK1-independent pattern-triggered immunity. Plant Cell 28, 1701-1721(2016).
- Igarashi, D., Tsuda, K. & Katagiri, F. The peptide growth factor, phytosulfokine, attenuates pattern-triggered immunity. *Plant J.* **71**, 194–204 (2012). 54.
- Mosher, S. et al. The tyrosine-sulfated peptide receptors PSKR1 and PSY1R 55 modify the immunity of Arabidopsis to biotrophic and necrotrophic pathogens in an antagonistic manner. Plant J. 73, 469-482 (2013).
- 56. Wang, J. et al. Allosteric receptor activation by the plant peptide hormone phytosulfokine. Nature 525, 265-268 (2015).
- 57. Postel, S. et al. The multifunctional leucine-rich repeat receptor kinase BAK1 is implicated in Arabidopsis development and immunity. Eur. J. Cell Biol. 89, 169–174 (2010).
- Sakamoto, T. et al. The tomato RLK superfamily: phylogeny and functional 58. predictions about the role of the LRRII-RLK subfamily in antiviral defense. BMC Plant Biol. 12, 229 (2012).



Extended Data Figure 1 | Expression profiles of LRR-RK ECDs produced as recombinant baits with the *Drosophila* S2 cells protein expression system. a–o, Western blot analyses of raw supernatants from S2 cells transfected with ECD expression vectors. Blots were cropped and arranged to match the phylogenetic tree of the LRR-RK gene family. The family subclasses and *Arabidopsis* gene initiative (AGI) identifiers are

indicated at the top. For lanes showing no obvious anti-V5 signals, a mild concentration of the S2 cell media and/or purification on protein-A-coated 96-well plates allowed for confirmation of expression and secretion of the ECDs. This experiment was conducted once with the full set of 200 ECDs. The expression of 130 independently expressed ECDs was tested one additional time with similar results.

RESEARCH LETTER



Extended Data Figure 2 | See next page for caption.



Extended Data Figure 2 | Calibration of the CSI^{LRR} screen conditions on ligand-dependent (FLS2-BAK1) and ligand-independent (BAK1-BIR4) interaction pairs. a, b, Western blot analyses of raw supernatants from S2 cells transfected with prey and bait expression vectors for the ECD of FLS2 (bait, western blot: anti-V5 antibody; prey, western blot: anti-Flag antibody). S2 cells left untreated (-) or treated with CuSO₄ (+). Days post transfection (dpt) are indicated on top. The experiment was repeated independently twice with similar results. c, Binding of the FLS2 ECD to the protein-A-coated 96-well plates. A fourfold dilution $(4\times)$ of the insect cell medium containing the ECD of FLS2 saturates the binding sites of protein-A-coated wells as indicated by immunoblots of the flow-through (FT). The experiment was repeated independently twice with similar results. d-f, As in a-c but for BAK1. The experiment was repeated independently twice with similar results. g, Plate interaction assays between the ECDs of BAK1 (prey) and FLS2 (bait) represented as cumulative absorbance (Abs 650 nm) over 18 h. Dots represent individual observations at each hour from five technical replicates. Box plots display the first and third quartiles, split by the median (red line); whiskers extend to include the maximum and minimum values. The presence of flg22 (+) in fourfold-diluted CSI^{LRR} screening conditions weakly promotes the interaction between the two ECDs. h, Technical replicates and box plots are as in g, but with BAK1 (bait) and FLS2 (prey). i, Technical replicates and box plots are as in g but with BAK1 (prey eightfold diluted) and FLS2 (bait fourfold diluted). In these conditions, the binding between the ECDs of BAK1 and FLS2 is largely enhanced by the presence of flg22 (+), indicating that the proteins produced in our expression system can interact in a ligand-dependent manner and are thus functional. j, Technical replicates and box plots as in g, but using a prey variant of BAK1 that can no longer pentamerize owing to the deletion of the COMP domain (BAK1 mono-prey). Binding between the two ECDs is still observed, but at a reduced level, thus indicating the importance of the pentamerization motif for detecting transient and low affinity interactions

in the absence of ligand. k, l, Binding of FLS2 and BAK1 ECDs to protein-A-coated 96-well plates (as indicated by immunoblots of the flow-through) when proteins are produced from S2 cells growing either at 21 °C or 27 °C. Immunoblots show a slight increase in protein production at 27 °C with similar binding capacities to the protein-A-coated plate. The protein expression levels at the two temperatures were assessed more than three times with similar results. The plate saturation experiment for proteins produced at 27 °C was conducted once. m, Plate interaction assays between BAK1 (prev) and FLS2 (bait) (in fourfold-diluted conditions) represented as cumulative absorbance (Abs 650 nm) over a 150-min time course. Dots represent individual observations made every 10 min from four technical replicates. Box plots as in g. Although slightly more abundant, proteins produced at 27 °C do not interact as well when produced at 21 °C. Protein expression for the CSI^{LRR} screen was performed at 21 °C. n, The FLS2–BAK1 interaction is insensitive to changes in pH conditions. Left, the interaction between FLS2 (bait) and BAK1 (prey) was observed in the pH range from 5.5 to 7.5. This experiment was conducted once. Right, plate interaction assays between BAK1 (prey) and FLS2 (bait) (in fourfold-diluted conditions) represented as cumulative absorbance over a 3-h time course. Dots represent individual observations at each hour from one technical replicate. The CSI^{LRR} screen was performed at the pH of the conditioned S2 cells supernatant (~pH 7.5). o, Plate interaction assays between BAK1 (as mono-prey (blue dots) or penta-prey (black dots)) and BIR4 represented as cumulative absorbance over a 3-h time course. Dots represent individual observations at each hour from one technical replicate. This experiment was conducted once. The data indicate that the pentamerization of the prey is a key requirement for enhancing the interaction detection sensitivity, without disrupting the functionality of the ECDs. BAK1 and BIR4 are ligand-independent interaction partners and the screening conditions used are also appropriate to detect this interaction.



Extended Data Figure 3 | Comparison of the primary and retest screens parameters. a, Geometric mean of the normalized absorbance values for the HCI (red dots) and LCI (yellow dots) obtained from the primary screen (CSI), the validation screen (retest) and the negative controls (NC) associated with the two screens. n denotes numbers of bidirectional interactions: HCI CSI (n = 567), HCI retest (n = 567), LCI CSI (n = 248), LCI retest (n = 248), and NC (n = 618). The box plots contain the first and third quartiles, split by the median (yellow or red lines indicated by the arrow on the left of the boxes); whiskers extend to include the maximum and minimum values. Statistical significance was determined using unbalanced one-way ANOVA by Tukey's HSD for all pairwise comparisons. Datasets with the same letter are indistinguishable at >95% confidence. b, Plots of a linear regression for the entire set of normalized absorbance values obtained from the retest screens (absorbance retest; y axis) and the corresponding values from the from the primary screen (absorbance CSI; x axis). The thick, straight red line is the linear regression that best describes the entire set of data points (Spearman's r = 0.7696; indicated on top). The fine red dashed lines represent the 95%

confidence intervals of the regression. n = 815 bidirectional interactions. c, Comparison of the geometric mean of normalized absorbance values for selected interactions. Values from the primary screen (absorbance CSI; y axis) and the validation screen (absorbance retest; x axis) are shown for the LCI set (yellow dots) and for 20 interactions selected at random from the HCI set (red dots). The number of interactions shown for each set was selected to approach the numbers present in the entire interaction search space. The red lines show the absorbance values corresponding to the FLS2-BAK1 interaction in both screens. d, Retest assay performance parameters interpreted within the performance window measured by positive reference set (PRS) and LCI calibration. To estimate the reliability of the estimates provided by the retest, the observed rate of interactions found in the HCI and LCI sets were used for a Monte Carlo simulation. n = 100,000 independent sets of observations selected at random from these populations, with the number of observations equal to the number present in the retest sets. These values were used to calculate the mean and s.d. of the samplings, which are presented as error bars.



Extended Data Figure 4 | **Characterization of BR11 interaction partners. a**, qPCR analyses showing altered gene expression in T-DNA lines targeting the interaction partners of BR11 (Fig. 1b). Genotypes are indicated. Relative expression levels were calculated and *ACTIN* was used as reference gene to control for cDNA amount in each reaction. The box plots contain the first and third quartiles, split by the median; whiskers extend to include the maximum and minimum values. n = 4 biologically independent mRNA samples for all genotypes, except for *bak1-4*, *skm1* and *sobir1* where n = 3. Statistical significance was estimated by an unpaired two-sided *t*-test and is indicated on top of the boxes: *erl2* **P*=0.0012, *fir*

 $*P = 5.3508 \times 10^{-6}$, $bak1-4*P = 3.08212 \times 10^{-7}$, $bam3*P = 1.9378 \times 10^{-5}$, serk4*P = 0.0108, $hsl2*P = 2.06945 \times 10^{-5}$, sark*P = 0.0259, rlk $*P = 2.12971 \times 10^{-10}$, $rul1*P = 7.49918 \times 10^{-5}$, $srf4*P = 3.08212 \times 10^{-7}$, $skm1*P = 5.5911 \times 10^{-6}$, sobir1*P = 0.0001. ns, not significant. **b**, T-DNA insertions targeting the HCI (top interactions) and LCI (bottom interaction) partners of BRI1. Morphology of representative seedlings grown for 7 days in the absence (NT) or presence (BL) of 500 nM brassinolide, the most potent brassinosteroid. Genotypes are indicated. The experiment was conducted six times with similar results.



Extended Data Figure 5 | Characterization of FLS2 interaction partners. a, qPCR analyses showing altered gene expression in T-DNA lines targeting the interaction partners of FLS2 (Fig. 1c). Genotypes are indicated. Relative expression levels were calculated and ACTIN was used as reference gene to control for cDNA amount in each reaction. n = 9 biologically independent mRNA samples for all tested genotypes. Statistical significance was estimated by an unpaired two-sided t-test: mik1 $P = 8.17192 \times 10^{-6}$, pskr1 P = 0.007, pepr2 P = 0.007, at3g14840 $*P = 0.005, at2g01210 *P = 0.0032, pepr1 *P = 1.16519 \times 10^{-5}, fei2$ *P = 0.005, *nik3* *P = 0.0015. **b**, Oxidative burst represented as total photon counts, triggered by 1 µM flg22 in wild type (black) and mutant lines targeting the HCI (top; red) and LCI (bottom, yellow) partners for FLS2. Genotypes are indicated. Dots represent individual observations from four independent experiments. n denotes numbers of biologically independent leaf discs: WT (n = 36), mik1 (n = 36), fls2 (n = 28), pskr1 (n = 27), pepr2 (n = 38), at3g46350 (n = 39). Statistical significance was determined using linear mixed effect modelling, and symbols indicate the results of a post hoc unpaired two-sided *t*-test corrected with the Holm method for multiple testing: $mik1 * P = 4.32 \times 10^{-2}$, $fls2 * P = 1 \times 10^{-15}$ **c**, As in **b**, except: WT (*n* = 32), *fls2* (*n* = 27), *bak1* (*n* = 39), *at3g14840* (n=33), at2g01210 (n=38), pepr1 (n=40). $bak1 * P = 1 \times 10^{-15}$, fls2* $P = 1 \times 10^{-15}$. d, As in b and c, except: WT (n = 43), fls2 (n = 29), bam3

(n = 33), fir (n = 39), srf9 (n = 32), fei2 (n = 45), nik3 (n = 32). fir * $P = 1.38 \times 10^{-3}$, fls2 * $P = 1.2 \times 10^{-15}$, nik3 * $P = 1.38 \times 10^{-3}$. The ROS burst assays in **b**-**d** were performed on independent plates (set number) and every plate contained wild type and *fls2* controls, as well as randomly assigned mutant lines. e, flg22-induced peroxidase (POX) assay in wildtype (black bar) and mutant lines targeting the HCI (top interactions; red) and LCI (bottom interactions, yellow) partners for FLS2. Genotypes are indicated. Leaf disks from 4-week-old plants were treated with water (NT) or 1 µM flg22 (T). The level of flg22-induced POX was normalized to the corresponding non-treated control. The level of POX present in the wild type was set to 100 for easier interpretation. n denotes numbers of biologically independent leaf discs from two independent experiments: WT (n = 44), mik1 (n = 10), fls2 (n = 17), bak1 (n = 31), bam3 (n = 42), *srf*9 (*n* = 18), *fir* (*n* = 55), *pskr1* (*n* = 24), *pepr2* (*n* = 12), *at3g46350* (n = 36), at3g14840 (n = 12), at2g01210 (n = 18), pepr1 (n = 12), fei2 (n = 11), nik3 (n = 15). Statistical significance was estimated using a paired two-sided *t*-test for each genotype, corrected for multiple tests using the Holm–Bonferroni correction. $mik1 * P = 5.71 \times 10^{-4}$, fls2 * P = 0.046, bak1 *P = 0.0039, fir *P = 0.0048, pskr1 *P = 9.49×10^{-5} . All box plots contain the first and third quartiles, split by the median; whiskers extend to include the maximum and minimum values.



Extended Data Figure 6 | FIR regulates flg22-induced responses. a, Seedlings of the genotypes indicated on the bottom were treated with either water (NT) or flg22 (T) and changes in FRK1 transcript levels were quantified by qPCR analyses. Dots represent individual observations from three independent experiments. *n* denotes numbers of biologically independent mRNA samples: WT (n = 9 (NT), n = 9 (T)), fir (n = 9, n = 9) and *fls2* (n = 6, n = 6). Statistical significance was determined using linear mixed effect modelling followed by comparison of each genotype to the wild-type control using unpaired two-sided *t*-test followed by multiple testing correction using the Holm method. *fir* $*P = 1.42 \times 10^{-7}$, *fls2* * $P = 4 \times 10^{-16}$. **b**, Growth of *Pto* DC3000 on the genetic backgrounds indicated at the bottom of the chart. Four-week-old plants were infiltrated with $10^5\,cfu\,ml^{-1}$ in the absence (black bars) or presence (grey bars) of $1\,\mu M$ flg22. The number of bacteria per area of leaf (cfu ml^{-1}) was plotted on a log₁₀ scale for day 0 (open bars) and day 3 (closed bars). Dots represent individual observations from two independent experiments. n denotes numbers of samples, each including 4 biologically independent leaf discs. For day 0, WT (n=6), fir (n=6), fls2 (n=6); for day 3, WT (n=6), fir

(n=6), fls2 (n=6); for day 3 + flg22, WT (n=6), fir (n=6), fls2 (n=6). Statistical significance for bacterial growth was estimated by two-way ANOVA. A third experiment performed at an inoculum of 10⁶ cfu ml⁻¹ corroborated these results. c, Morphology of 7-day-old seedlings grown in the absence (-) or presence (+) of 1 μ M flg22. Genotypes are indicated. The experiment was conducted twice with similar results. d, Primary root length (cm) from seedlings grown in the presence (T) or absence (NT) of 1 μ M flg22. Fold changes are T/NT ratios. Dots represent individual observations from two independent experiments. *n* denotes the following numbers of biologically independent roots: WT (n = 32 (NT), n = 36 (T)), fir (n = 34 (NT), n = 32 (T)), fls2 (n = 27 (NT), n = 26 (T)). Statistical significance for two biological replicates was determined using linear mixed effect modelling followed by comparison of each genotype to the wild-type control using unpaired two-sided *t*-test followed by multiple testing correction using the Holm method. fir $*P = 2.02 \times 10^{-6}$, fls2 $*P = 2.02 \times 10^{-6}$. All box plots display the first and third quartiles, split by the median; whiskers extend to include the maximum and minimum values.

RESEARCH LETTER



Extended Data Figure 7 | CSI^{LRR} network representation and table of nodes with their corresponding identification numbers or acronyms. The network construction and other features are the same as shown in Fig. 2b. The nodes surrounded by white halos are articulation points. The numbers in each node corresponding to the ECD of each LRR-RK are shown in the table.



Extended Data Figure 8 | See next page for caption.



Extended Data Figure 8 | Characterization of independent apex mutant and 35S::APEX transgenic lines. a, Top, rosette morphology of 4-weekold wild-type, apex-1 and apex-2, and apex-3 knockdown lines grown under long-day photoperiod at 22 °C. Genetic backgrounds are indicated. No obvious changes in rosette morphology are observed. The experiment was conducted three times with similar results. Bottom, qPCR analyses showing fold reduction of APEX transcripts in the independent mutant lines. Relative expression levels were calculated and ACTIN was used as reference control gene. Dots represent individual observations from three independent experiments. n = 9 biologically independent mRNA samples for each genotype. Statistical significance was determined using linear mixed effect modelling followed by comparison of each genotype to the wild-type control using unpaired two-sided *t*-test followed by multiple testing correction using the Holm method. *apex-1* * $P = 6 \times 10^{-16}$, *apex-2* $*P = 5.33 \times 10^{-15}$, apex-3 $*P = 6 \times 10^{-16}$. **b**, Top, rosette morphology of 3-week-old wild type and 35S::APEX lines 1 and 2 grown under long-day photoperiod at 22 °C. Genetic backgrounds are indicated on the top. Rosettes of 35S:: APEX lines are slightly larger than WT under long-day photoperiod at 22 °C. The experiment was conducted three times with similar results. Middle: Quantitative real-time PCR analyses showing fold induction of the APEX transgene in the overexpression lines used in this study. Relative expression levels were calculated and ACTIN was used as reference gene to control for cDNA amount in each reaction. Dots represent individual observations from two independent experiments. n = 6 biologically independent mRNA samples for each genotype. Statistical significance was determined using linear mixed effect modelling followed by comparison of each genotype to the WT control using an unpaired two-sided *t*-test followed by multiple testing correction using the

Holm method and is indicated on top of the boxes: 35S::APEX line 1 $*P = 3.38 \times 10^{-14}$, 35S::APEX line 2 $*P = 7.77 \times 10^{-14}$. Bottom, detection of APEX-YFP in stable transgenic T₃ lines by western blot using an anti-GFP antibody. c, Modulation of BRI1 signalling by APEX gene dosage. Morphology of representative seedlings corresponding to Fig. 4a. Genotypes are indicated. The experiment was conducted over three times with similar results. d, Hypocotyl length ratios of seedlings grown in the presence (T) or absence (NT) of 500 nM brassinolide (BL). Genotypes are indicated. Dots represent individual observations from three independent experiments. n denotes numbers of biologically independent hypocotyls. WT (n = 43 (NT), n = 33 (T)), apex-1 (n = 31, n = 35), apex-2 (n = 32, n = 33), apex-3 (n = 39, n = 38), bri1 (n = 28, n = 32). Statistical significance was determined using linear mixed effect modelling followed by comparison of each genotype to the wild-type control using unpaired two-sided *t*-test followed by multiple testing correction using the Holm method. $apex-1 * P = 2.53 \times 10^{-14}$, $apex-2 * P = 1.10 \times 10^{-5}$, apex-3* $P = 1.55 \times 10^{-12}$, bri1 * $P = 8 \times 10^{-16}$. **e**, flg22-induced oxidative bursts represented as total photon counts over 40 min. Genetic backgrounds are indicated. Dots represent individual observations from three independent experiments. n denotes numbers of biologically independent leaf discs: WT (n = 31), apex-1 (n = 19), apex-2 (n = 23), apex-3 (n = 25), fls2 (n = 15). Statistical significance was determined using linear mixed effect modelling followed by comparison of each genotype to the wild-type control using an unpaired two-sided *t*-test followed by multiple testing correction using the Holm method. *apex-1* * $P = 2.99 \times 10^{-3}$, *apex-2* $*P = 2.84 \times 10^{-2}$, apex-3 $*P = 2.84 \times 10^{-2}$, fls2 $*P = 8 \times 10^{-16}$. All box plots display the first and third quartiles, split by the median (red line); whiskers extend to include the maximum and minimum values.



Extended Data Figure 9 | **Modulation of brassinosteroid signalling by AT5G51560. a**, Morphology of representative seedlings grown for 7 days in the absence (NT) or presence (BL) of 500 nM brassinolide. Genotypes are indicated. The experiment was conducted twice with similar results. **b**, Hypocotyl length fold changes corresponding to **a**. Genotypes are indicated. Dots represent individual observations from two independent experiments. *n* denotes numbers of biologically independent hypocotyl: WT (n = 39 (NT), n = 29 (T)), at5g51560 line 1 (n = 36 (NT), n = 26 (T)),

at5g51560 line 2 (n = 39 (NT), n = 34 (T)), *bri1* (n = 25 (NT), n = 27 (T)). Box plots display the first and third quartiles, split by the median; whiskers extend to include the maximum and minimum values. Statistical significance was determined using linear mixed effect modelling followed by comparison of each genotype to the wild-type control using an unpaired two-sided *t*-test followed by multiple testing correction using the Holm method. *at5g51560* line 1 * $P = 3.75 \times 10^{-6}$, *at5g51560* line 2 * $P = 2.26 \times 10^{-12}$, *bri1* * $P = 6 \times 10^{-16}$.

natureresearch

Youssef Belkhadir Corresponding author(s): Manuscript# Nature 2016-09-13425F

Initial submission 📃 Revised version

K Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

Sample size

1

т.	Sumple Size	
	Describe how sample size was determined.	No statistical methods were used to predetermine sample sizes. Sample sizes were chosen as large as possible while still practically doable in terms of data collection. Adequate statistics has been applied throughout the manuscript in order to make sure that the observed effects are significant given the reported sample size.
2.	Data exclusions	
	Describe any data exclusions.	Unless otherwise noted, all analyses involved the data being combined, and outliers removed using the ROUT method, as implemented in GraphPad PRISM 7.0 (Q = 0.1%) (see Supplementary Text 1). For Hypocotyl and root length measurements assays only seedlings that germinated synchronously were considered for measurement and statistical analysis.
3.	Replication	
	Describe whether the experimental findings were reliably reproduced.	Except when caused by technical issues (e.g. plate contamination), all attempts at replication were successful. Unless otherwise noted, in all analyses the biological replicates have been combined and analyzed by linear mixed effects models as per suggestion of the referees.
4.	Randomization	
	Describe how samples/organisms/participants were allocated into experimental groups.	Not relevant as grouping was not applied.
5.	Blinding	
	Describe whether the investigators were blinded to group allocation during data collection and/or analysis.	No investigator blinding was applied during data acquisition or analyses

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a Confirmed

] 🔀 The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

A statement indicating how many times each experiment was replicated

The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)

- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- K A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

► Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

The following software were used:
i- R programming environment version 3.4.2:igraph package for the
implementation of the WalkTrap and PageRank algorithms, Ime4 package for
implementation of the linear mixed effect models, ImerTest package for
determination of statistical significance, multcomp package for pairwise
comparisons.
ii- GraphPad PRISM 7.0
iii-ImageJ bundled with Java 1.8.0_112
iv-No computer code was used to generate data.
v-We have developed a software that we named "PLATERO" that allows one to
collate data generated by a 96 well plate-reader into a suitable excel document for
subsequent analysis. PLATERO is either available on request or available for direct

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

download through GitHub.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company. There are no restrictions

ture research | life sciences reporting summar

9. Antibodies

ercially available he Arabidopsis ERK4 are required Plant Cell 2011;
S2). This is a ems. The uired from
enticated.
use facility.
AC database.

> Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Research animals were not used in the study.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

Human participants were not used in the study.

CORRECTION

https://doi.org/10.1038/s41586-018-0268-y

Publisher Correction: An extracellular network of *Arabidopsis* leucine-rich repeat receptor kinases

Elwira Smakowska-Luzan, G. Adam Mott, Katarzyna Parys, Martin Stegmann, Timothy C Howton, Mehdi Layeghifard, Jana Neuhold, Anita Lehner, Jixiang Kong, Karin Grünwald, Natascha Weinberger, Santosh B. Satbhai, Dominik Mayer, Wolfgang Busch, Mathias Madalinski, Peggy Stolt-Bergner, Nicholas J. Provart, M. Shahid Mukhtar, Cyril Zipfel, Darrell Desveaux, David S. Guttman & Youssef Belkhadir

Correction to: *Nature* https://www.nature.com/articles/nature25184, published online 10 January 2018.

In this Letter, an incorrect version of the Supplementary Information file was inadvertently used, which contained several errors. The details of references 59–65 were missing from the end of the Supplementary Discussion section on page 4. In addition, the section 'Text 3. Y2H on ICD interactions' incorrectly referred to 'Extended Data Fig. 4d' instead of 'Extended Data Fig. 3d' on page 3. Finally, the section 'Text 4. Interaction network analysis' incorrectly referred to 'Fig. 1b and Extended Data Fig. 6' instead of 'Fig. 2b and Extended Data Fig. 7' on page 3. These errors have all been corrected in the Supplementary Information.