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## The receptor MIK2 interacts with the kinase RKS1 to control quantitative disease resistance to *Xanthomonas campestris*

Florent Delplace,\*<sup>1</sup> Carine Huard-Chauveau, Fabrice Roux,<sup>1</sup> Dominique Roby\*

Laboratoire des Interactions Plantes-Microbes Environnement (LIPME), INRAE, CNRS, Université de Toulouse, 31326 Castanet-Tolosan, France

\*Author for correspondence: [florent.delplace@inrae.fr](mailto:florent.delplace@inrae.fr) (F.D.), [dominique.robey\\_descazal@gmail.com](mailto:dominique.robey_descazal@gmail.com) (D.R.)The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (<https://academic.oup.com/plphys/pages/General-Instructions>) is Carine Huard-Chauveau.

### Abstract

Molecular mechanisms underlying qualitative resistance have been intensively studied. In contrast, although quantitative disease resistance (QDR) is a common, durable, and broad-spectrum form of immune responses in plants, only a few related functional analyses have been reported. The atypical kinase Resistance related kinase 1 (RKS1) is a major regulator of QDR to the bacterial pathogen *Xanthomonas campestris* (Xcc) and is positioned in a robust protein–protein decentralized network in *Arabidopsis thaliana*. Among the putative interactors of RKS1 found by yeast two-hybrid screening, we identified the receptor-like kinase MDIS1-interacting receptor-like kinase 2 (MIK2). Here, using multiple complementary strategies including protein–protein interaction tests, mutant analysis, and network reconstruction, we report that MIK2 is a component of RKS1-mediated QDR to Xcc. First, by co-localization experiments, co-immunoprecipitation (Co-IP), and bimolecular fluorescence complementation, we validated the physical interaction between RKS1 and MIK2 at the plasma membrane. Using *mik2* mutants, we showed that MIK2 is required for QDR and contributes to resistance to the same level as RKS1. Interestingly, a catalytic mutant of MIK2 interacted with RKS1 but was unable to fully complement the *mik2-1* mutant phenotype in response to Xcc. Finally, we investigated the potential role of the MIK2–RKS1 complex as a scaffolding component for the coordination of perception events by constructing a RKS1–MIK2 centered protein–protein interaction network. Eight mutants corresponding to seven RKSs in this network showed a strong alteration in QDR to Xcc. Our findings provide insights into the molecular mechanisms underlying the perception events involved in QDR to Xcc.

### Introduction

In response to pathogens, plants have developed complex resistance mechanisms that are either constitutively expressed or induced after a pathogen attack (Glazebrook 2005; Panstruga et al. 2009). Quantitative disease resistance (QDR) is the predominant form of resistance in crops and natural populations. QDR is characterized by a polygenic determinism conferring partial and generally broad-spectrum resistance (Poland et al. 2009; Roux et al. 2014b). For this reason, the molecular mechanisms underlying QDR remain poorly understood. However, the recent cloning of a limited number of QDR genes underlying resistance quantitative trait loci (QTLs) revealed a broad range of molecular functions (Delplace et al. 2022; Demirjian et al. 2023a). Only a very few QDR genes encode NLRs (nucleotide-binding leucine-rich repeat receptors) identified as R genes in the context of qualitative resistance (Broglie et al. 2006; Staal et al. 2006; Fukuoka et al. 2014; Demirjian et al. 2023b). On the other hand, several QDR genes encode different types of receptors including receptor-like kinases (RLKs), signaling components such as kinases, and diverse metabolic functions (Corwin and Kliebenstein 2017; Pilet-Nayel et al. 2017; Nelson et al. 2018). This diversity of molecular functions together with the partial resistance conferred by QDR genes suggests that QDR results from a complex network integrating

diverse pathways in response to multiple pathogenic determinants (Roux et al. 2014b). This suggests in turn that (i) individual QDR genes may confer resistance to multiple pathogens (Nelson et al. 2018; Kanyuka and Rudd 2019), and (ii) multiple receptors act together to perceive diverse pathogen determinants (Nguu et al. 2022). However, a limited number of receptors have been identified in the context of QDR in various plant–pathogen interactions.

In plants, RLKs play an essential role in environmental signal perception, including pathogen detection at early stages of infection. RLKs detect different pathogen epitopes. The transmembrane RLK FLAGELLIN SENSING 2 (FLS2) is implicated in the detection of flg22, a conserved peptide of bacteria flagellin (Gómez-Gómez and Boller 2000; Bauer et al. 2001). The EF-Tu receptor (EFR) is involved in the perception of the peptide elf18, the N-terminal peptide of EF-Tu (Kunze et al. 2004; Zipfel et al. 2006). However, the number of distinct perception systems with specificity for microbe-associated molecular patterns (MAMPs) is difficult to estimate and a limited number of RLKs have been described to recognize pathogen signatures (Tang et al. 2017). Recently, a complex and dynamic network of leucine-rich repeat (LRR)–RLKs interactions has been described in *Arabidopsis* (Smakowska-Luzan et al. 2018). The association of LRR–RLKs in heterodimers might

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participate in a large detection of diverse epitopes involved in various plant processes. RK-dependent recognition events converge into receptor hubs and overlapping immune signaling components can be recruited (Holton et al. 2015; Adachi and Tsuda 2019). These Rks take place in plasma membrane protein complexes including other Rks, receptor proteins (RPs), kinases, or pseudokinases (Boudeau et al. 2006; Monaghan and Zipfel 2012) and play a central role in the modulation of plant immunity. Some pathogen effectors have been shown to physically interact with essential signaling components to inhibit fast information spreading (Ahmed et al. 2018). In the same vein, complex associations between some NLRs have been observed in homodimers and heterodimers (Wróblewski et al. 2018; Contreras et al. 2023), and most cell surface and intracellular immune receptors appear to engage other receptors including ligand-binding receptors and transducer co-receptors (Wu et al. 2018). In summary, pathogen detection is not limited to a ligand perception event and receptors are organized in complex networks involving multiple perception events, in order to recognize a large variety of pathogen determinants. However, only a few of such ligand-binding receptors or receptor complexes have been identified and reported in the literature.

In the plant–pathogen interaction between *Arabidopsis thaliana* and the vascular bacterium *Xanthomonas campestris* pv. *campestris* (Xcc), RKS1 was identified to confer QDR to Xcc (Huard-Chauveau et al. 2013). RKS1 encodes an atypical kinase lacking some critical domains in the kinase catalytic core (Huard-Chauveau et al. 2013; Roux et al. 2014a). Atypical kinases (or pseudokinases) have been described as important regulators of signaling networks (Blaum et al. 2014; Reiterer et al. 2014). Recently, we reported a highly interconnected and decentralized RKS1-dependent protein–protein network, which is largely distinct from effector-triggered immunity (ETI) and pattern-triggered immunity (PTI) responses already characterized in *A. thaliana* (Delplace et al. 2020). From this network, we identified a signaling subnetwork that includes Rks not previously described in the context of plant immunity for most of them. Thus, functional analysis of Rks associated with RKS1 could provide a powerful tool for deciphering molecular mechanisms involved in the early signaling events of QDR. In this study, we report that the LRR-RK MDIS1-interacting receptor-like kinase 2 (MIK2) interacts with RKS1 and that MIK2 and RKS1 co-localized at the plasma membrane. We also show that *mik2* mutants exhibit an increased susceptibility to Xcc as compared to the wild type and that MIK2 catalytic activity is required for QDR to Xcc. In addition, the double mutant *mik2-1/rks1-1* showed a similar level of resistance against Xcc than the single mutants, indicating that they probably belong to the same pathway. Interestingly, MIK2 was recently demonstrated as a serine-rich endogenous peptide (SCOOP) perceiving RK (Hou et al. 2021; Rhodes et al. 2021). Accordingly, we found that MIK2 is positioned in a putative receptors-interacting network. Insertional mutants for some of these Rks showed increased susceptibility to Xcc, suggesting that they participate with MIK2 to Xcc perception. Our data support the hypothesis that MIK2 and RKS1 act as a scaffolding hub in an RK network to centralize and modulate pathogen determinant perception for QDR signaling and activation.

## Results

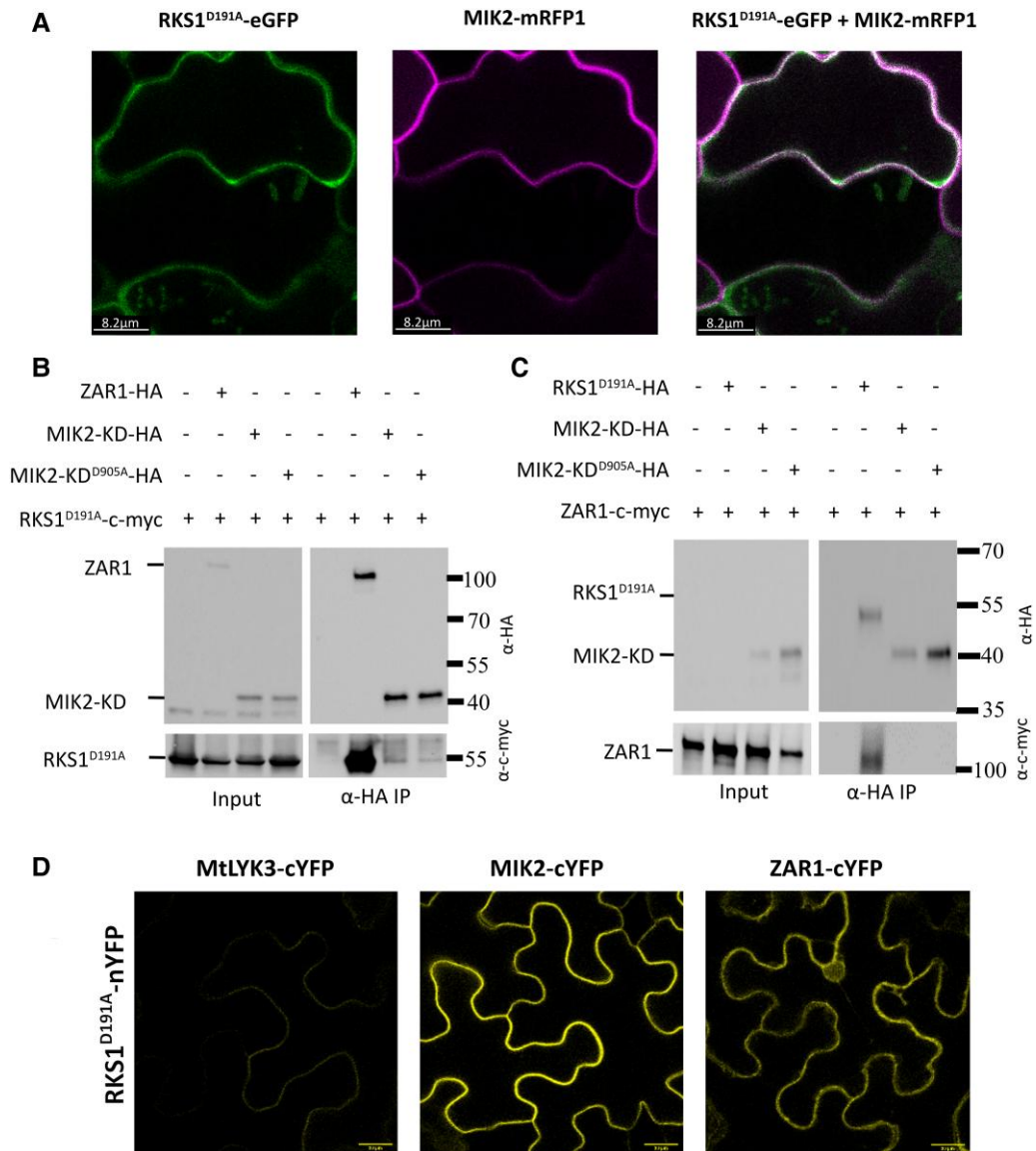
### RKS1 physically interacts with the kinase domain of MIK2

Using a yeast two-hybrid screen and a mutated version of RKS1 (RKS1<sup>D191A</sup>) as a bait against a cDNA library generated from leaves

inoculated by the strain Xcc147 (Froidure et al. 2010), we identified among 43 candidate proteins (Delplace et al. 2020), a fragment of the kinase domain of MIK2 as a putative interactor of RKS1. We investigated the interaction between RKS1 and MIK2 using several strategies. First, to test whether RKS1 and MIK2 might be localized in the same cellular compartment, constructs using the fluorescent C-terminal tags eGFP and mRFP1, respectively, fused to the full-length sequences of RKS1<sup>D191A</sup> and MIK2, were co-transfected into *Arabidopsis* seedlings. As previously reported (Delplace et al. 2020), RKS1<sup>D191A</sup>-eGFP was detected in the plasma membrane, the cytoplasm, and the nucleus. MIK2 was detected only in the plasma membrane (Fig. 1A). An overlap between eGFP and mRFP1 signals indicates a close subcellular localization of MIK2 and RKS1 in the plasma membrane. Second, co-immunoprecipitation assays were conducted using RKS1<sup>D191A</sup> fused to a c-myc tag and the kinase domain of MIK2 (MIK2-KD, amino acids 775 to 1045, including the cDNA fragment identified in Y2H) fused to a HA (Hemagglutinin) tag. After immunoprecipitation with an anti-HA antibody, RKS1<sup>D191A</sup>-c-myc was detected when incubated with MIK2-KD-HA (Fig. 1B). ZAR1-HA, used as a positive control of interaction with RKS1 (Wang et al. 2015) and RKS1<sup>D191A</sup>-c-myc, was not detected after immunoprecipitation with tag-HA, indicating the specific nature of interaction between MIK2 and RKS1 (Fig. 1C). An immunoprecipitation of RKS1<sup>D191A</sup>-HA with an anti-HA antibody was also able to pull down MIK2 full-length protein with a c-myc tag (Supplementary Fig. S1). Third, we performed bimolecular fluorescence complementation (BiFC) assays using the split YFP system. The N-terminal half of the yellow fluorescent protein was fused to RKS1<sup>D191A</sup> and the YFP C-terminal was fused to MIK2, ZAR1, or MtLYK3 (Lefebvre et al. 2010). When RKS1<sup>D191A</sup>-nYFP was co-transformed with MIK2-cYFP, YFP signals were observed in the plasma membrane in *N. benthamiana* leaves. Similar signals were observed in *A. thaliana* protoplasts, localized exclusively at the cell periphery and excluding chloroplasts (Supplementary Fig. S2). As a comparison, when RKS1-nYFP was co-transformed with ZAR1-cYFP, YFP signals were detected in the plasma membrane and the cytoplasm (Fig. 1D). An extremely weak YFP signal was observed in the plasma membrane with RKS1<sup>D191A</sup>-nYFP and the receptor MtLYK3-cYFP co-transformed in *N. benthamiana* leaves. Similar weak YFP signal was observed using co-transformation with the receptor MtLRR2-cYFP (Lefebvre et al. 2010) (Supplementary Fig. S3). These results collectively demonstrate that RKS1 is able to physically interact with the kinase domain of MIK2 in the plasma membrane.

### MIK2 partially controls RKS1-dependent QDR

To investigate the function of MIK2 in Xcc resistance, we characterized two T-DNA homozygous mutant lines for MIK2, i.e. *mik2-1* (GK-208H02, *mik2-3* in Coleman et al. 2020) and *mik2-2* (SALK\_061769, *mik2-1* in Rhodes et al. 2021) (GABI-Kat and SALK collection, Col-0 background). The flanking regions of the T-DNA insertion sites were sequenced and the T-DNA insertion sites were found in the first exon of MIK2, at +2015 bp and +2644 bp, respectively (Fig. 2A). Complementarily, we generated transgenic lines complemented by AtMIK2 in the *mik2-1* mutant background. MIK2 gene expression in *mik2* mutants and *mik2-1* complemented lines is presented in Supplementary Fig. S4. While 31% and 5% of residual MIK2 gene expression were found in *mik2-1* and *mik2-2* mutants, respectively, MIK2 gene expression was restored in the complemented *mik2-1*(35S::MIK2) #1 and #2 lines (629% and 57%), as compared to Col-0. *mik2* mutants and *mik2-1* complemented lines

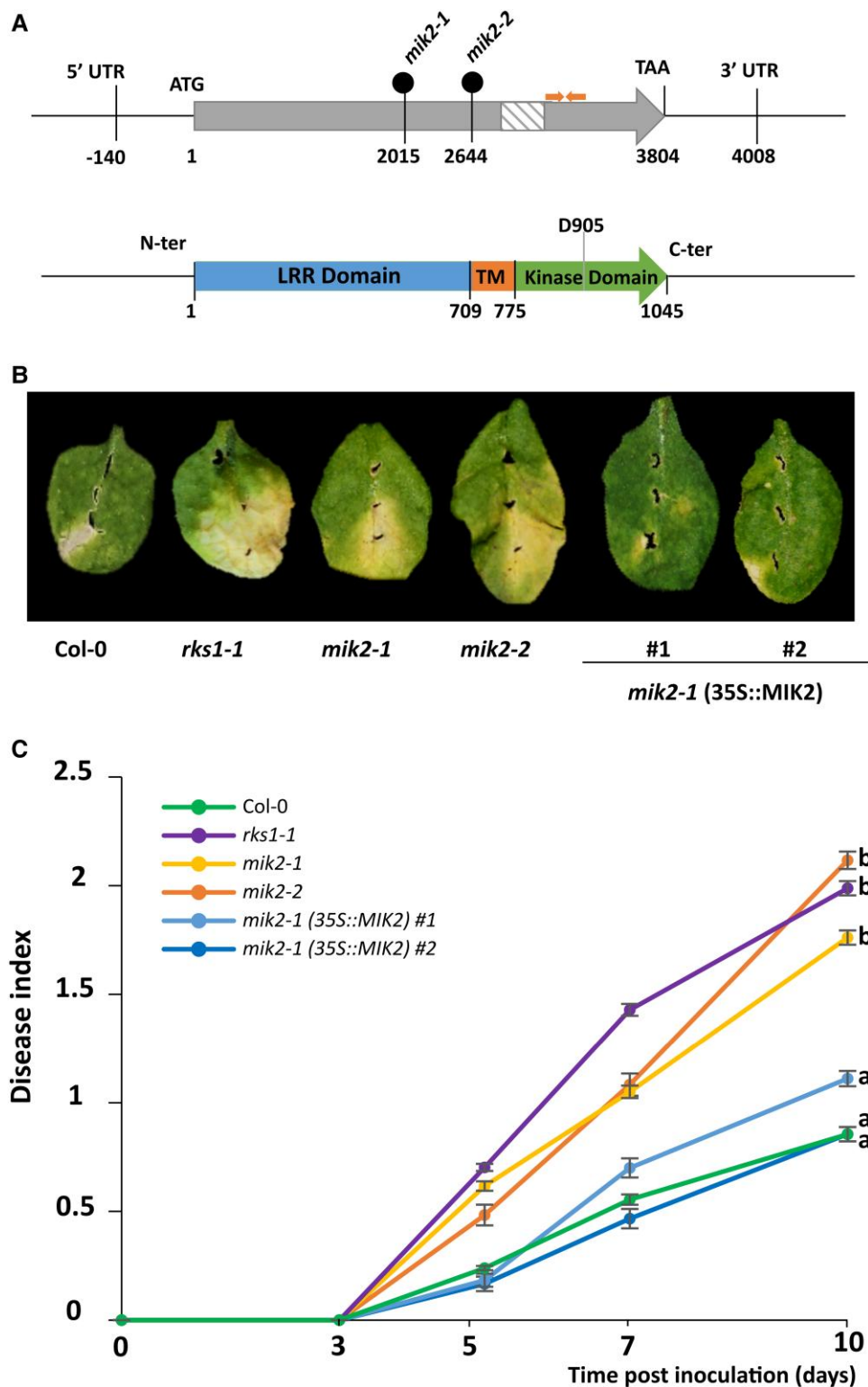


**Figure 1.** MIK2 physically interacts with RKS1 in the plasma membrane but not with ZAR1. **A)** Arabidopsis leaves were co-transformed by RKS1<sup>D191A</sup>-eGFP and MIK2-mRFP1 full length constructs. Green corresponds to GFP signal, purple corresponds to RFP signal and white, co-localization between GFP and RFP. The white bars represent the ladder scale. **B)** Co-immunoprecipitation experiments with protein extracts from *N. benthamiana* inoculated with the C58C1 *Agrobacterium* strain carrying ZAR1-HA, MIK2-KD-HA or MIK2-KD<sup>D905A</sup>-HA constructs, and with the C58C1 strain carrying RKS1<sup>D191A</sup>-c-myc. Input proteins were extracted and revealed by anti-HA and anti-c-myc HRP immunoblotting. Then, total proteins were subjected to anti-HA immunoprecipitation (α-HA IP), separated on a 4%–15% SDS-PAGE gel and detected by anti-HA and anti-c-myc immunoblotting. **C)** Co-immunoprecipitation experiments with protein extracts from *N. benthamiana* inoculated by the C58C1 *Agrobacterium* strain carrying RKS1<sup>D191A</sup>-HA, MIK2-KD-HA or MIK2-KD<sup>D905A</sup>-HA constructs in one hand, and in the other hand, with a strain carrying ZAR1-c-myc. Proteins were analyzed by immunoblotting as described in part **B)**. **D)** BiFC assay between RKS1 and MIK2, ZAR1, or MtLYK3. RKS1<sup>D191A</sup> was fused to nYFP and MIK2, ZAR1, and MtLYK3 were fused to cYFP. The indicated constructs were co-transformed into *N. benthamiana* leaves. The panel represents the YFP signal; bars in bottom right corner indicate the scale (20 µm).

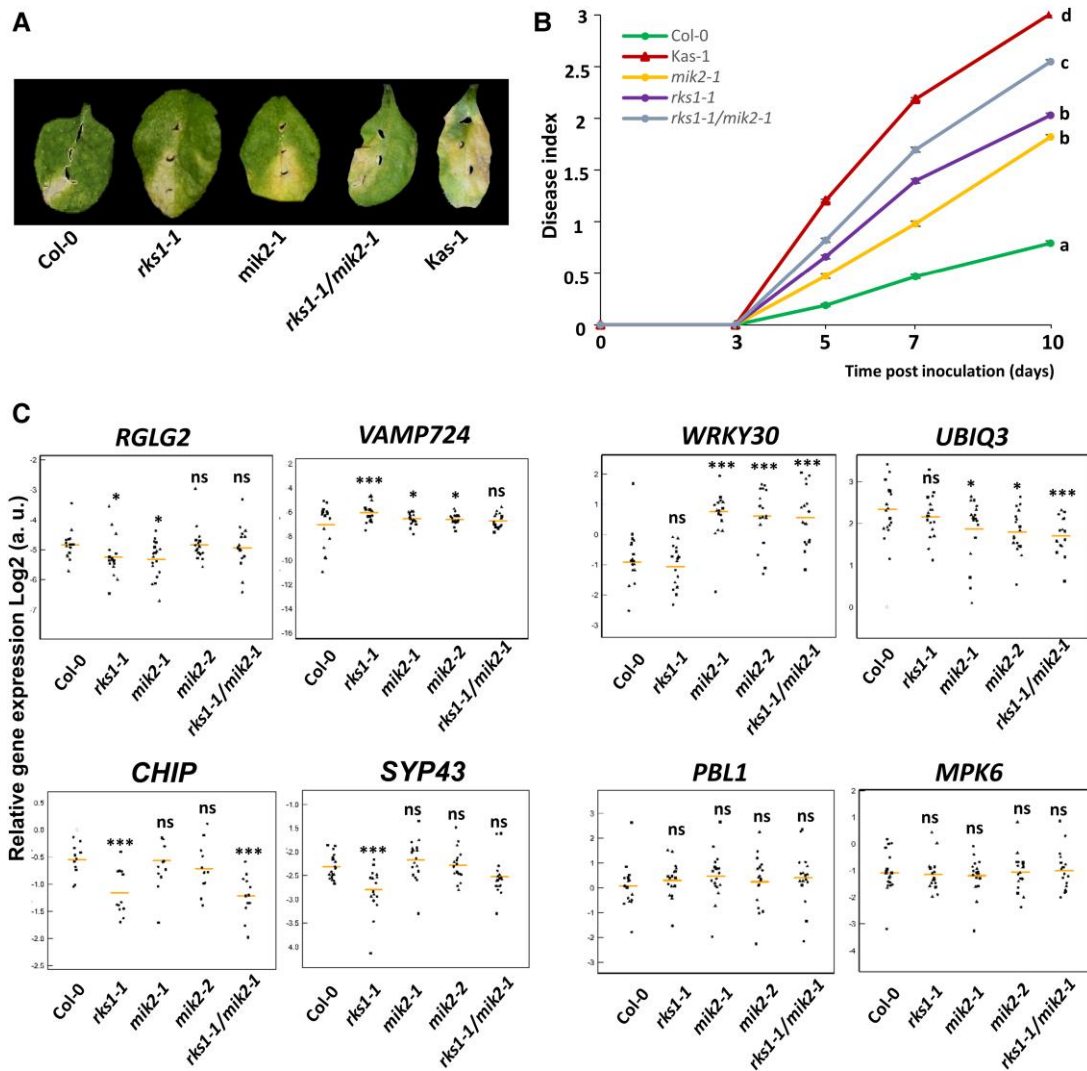
were then inoculated with a bacterial suspension of the strain Xcc568 and disease index evaluated at 3-, 5-, 7-, and 10-days postinoculation (Fig. 2B and C; Supplementary Fig. S5). *mik2* mutants showed a significant increased susceptibility compared to Col-0, and this increased susceptible phenotype was not statistically different from the *rks1-1* mutant phenotype. These results were confirmed by measurement of Xcc568 bacterial growth in planta (Supplementary Fig. S6). Complemented lines showed a similar resistance level than Col-0. These data confirm the implication of MIK2 in QDR to Xcc568.

To further explore the respective roles of MIK2 and RKS1 in the QDR, we generated the double mutant *rks1-1/mik2-1* and

phenotyped it in response to inoculation with Xcc568 (Fig. 3A and B; Supplementary Fig. S6). The double mutant was significantly more susceptible than Col-0, slightly more susceptible than the single mutants, and less susceptible than the susceptible reference accession Kashmir-1 (Kas-1). This result suggests that MIK2 might at least be partially involved in the RKS1 pathway mediating QDR to Xcc568. To investigate more precisely the respective roles of MIK2 and RKS1 in these pathways, we analyzed the gene expression of a total of 36 genes highly connected to RKS1 from the previously reconstructed RKS1-dependent gene network (Delplace et al. 2020) or described to be dependent from MIK2 expression (Hou et al. 2021). Expression of these genes was measured by



**Figure 2.** *Mik2* mutants are more susceptible to *Xcc568* inoculation. **A)** Schematic representation of the genomic organization of the *mik2* mutants and of the MIK2 protein. MIK2 consists in two exons (gray boxes) and one intron (hatched box). For *mik2-1* and *mik2-2*, the T-DNA is integrated in the first exon, respectively at the position 2015 and 2644. The orange arrows indicate the primers used for measurement of MIK2 gene expression. For the schematic representation of the MIK2 protein, blue represents the LRR domain; orange, the transmembrane domain; green, the kinase domain and D905 the aspartic amino acid involved in the kinase catalytic site. **B)** Disease symptoms were observed on leaves of *mik2* and *rks1* mutants, two *mik2-1* complemented lines with a 35S::MIK2 construct (#1 and #2) and Col-0 wild-type plants, 10 days postinoculation with a bacterial suspension adjusted to  $2.10^8$  cfu/mL. Images were digitally extracted for comparison. **C)** Time course evaluation of disease index after inoculation of *rks1-1* (purple), *mik2-1* (yellow), *mik2-2* (orange), *mik2-1 (35S::MIK2) #1* (light blue), *mik2-1 (35S::MIK2) #2* (dark blue), and the wild type Col-0 (green) with *Xcc568* under the same conditions as **B)**. Means and standard error of the mean (SEM) were calculated from 5 plants, 4 leaves/plant, and based on 3 independent experiments (60 values by point). Statistical tests were performed by comparing the disease index kinetics modeling differences, with a P-value threshold of 0.05. Different letters indicate significant differences in disease index kinetics among the lines.



**Figure 3.** MIK2 participates to the RKS1 dependent control of QDR in response to Xcc. **A)** Disease symptoms were observed on leaves of *mik2-1* and *rks1-1* mutants, the double mutant *rks1-1/mik2-1*, the susceptible accession *Kas-1* and the wild-type *Col-0*, 10 days after inoculation with a bacterial suspension of *Xcc568* adjusted to  $2.10^8$  cfu/mL. Images were digitally extracted for comparison. The *Col-0* and *mik2-1* images are the same as those in Fig. 2B. **B)** Time course evaluation of disease symptoms and index after inoculation of *mik2-1* (yellow), *rks1-1* (purple), *rks1-1/mik2-1* (gray), the susceptible accession *Kas-1* (red), and the wild type *Col-0* (green) with a bacterial suspension of *Xcc568* adjusted to  $2.10^8$  cfu/mL. Disease scores were observed at 3-, 5-, 7-, and 10-days post-inoculation. Means and SEM) were calculated from five plants based on six independent experiments (120 values by point). Statistical tests were performed by comparing the disease index kinetics modeling differences, with a P-value threshold of 0.05. Different letters indicate significant differences in disease index kinetics among the lines. **C)** Jitter plots illustrating RT-qPCR results of the expression profile of genes from the RKS1-dependent gene network (Delplace et al. 2020), 6 h after inoculation with *Xcc568* ( $2.10^8$  cfu/mL) in leaves of the wild type accession, *Col-0*, in *mik2-1* and *mik2-2* mutants, the *rks1-1* mutant and *rks1-1/mik2-1* double mutant. Dots correspond to the relative gene expression values adjusted for micro-environmental variation between experiments by retrieving the residuals after fitting a model only with the term “experiment” and then adding the general mean value of relative gene expression values to these residuals. Data from the first, second, and third experiments are represented by square, circle, and triangle symbols. Mean relative gene expression is represented by an orange segment. The *RGLG2* and *VAMP724* genes present an expression profile dependent on the presence of MIK2 and RKS1. The *WRKY30* and *UBIQ3* genes present an expression profile dependent on the presence of MIK2 and independent of RKS1. The *CHIP* and *SYP43* genes present an expression profile independent of the presence of MIK2 and dependent on RKS1. The *PBL1* and *MPK6* genes present an expression profile independent of the presence of MIK2 and RKS1. Tests for statistical differences with *Col-0* were performed with a GLM procedure in the R environment on three independent experiments with six plants/line/experiment. ns: nonsignificant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . A correction for the number of tests was performed to control the FDR at a nominal level of 5%. a. u., arbitrary units.

RT-qPCR in *Col-0* and the mutants *rks1-1*, *mik2-1*, *mik2-2*, *rks1-1/mik2-1*, 6 h after inoculation by *Xcc568* (Supplementary Table S1). A significant fraction of genes (44%) showed an expression dependent of the mutation *mik2* (either *mik2-2*, or both *mik2-1* and *mik2-2*). In this fraction, 31% showed an expression profile dependent of both mutations *rks1* and *mik2*. Several examples are presented in Fig. 3C. These results clearly support the hypothesis that MIK2 and RKS1 initiate common signaling pathways to control gene

expression. However, these common pathways appear to constitute only a fraction of the ones initiated by both proteins, in good agreement with the double mutant phenotype reported in Fig. 3A and B. Interestingly, MIK2 gene expression was significantly reduced in two independent RKS1 overexpressing lines (RKS1-OE1 and RKS1-OE2) (Huard-Chauveau et al. 2013; Delplace et al. 2020) (Supplementary Fig. S4B), suggesting that MIK2 gene expression might be regulated by an overexpression of RKS1.

## Catalytic activity of MIK2 is required for QDR to Xcc568

Taking in account that (i) MIK2 participates to the RKS1 dependent QDR in response to Xcc, and (ii) RKS1 interacts with the kinase domain of MIK2, we tested whether these MIK2 functions depend on its kinase catalytic activity. For this purpose, an inactive catalytic mutant version of MIK2 full-length protein, MIK2<sup>D905A</sup>, affected in the putative active HRD site of the MIK2 kinase domain, was generated. Kinase assays using MIK2 and MIK2<sup>D905A</sup> showed that MIK2 was able to autophosphorylate and that the aspartic acid residue in position 905 was required for MIK2 autophosphorylation activity (Fig. 4A). First, the catalytic mutated version of the kinase domain of MIK2, MIK2-KD<sup>D905A</sup>-HA, was able to interact with RKS1<sup>D191A</sup> fused to the c-myc tag, as shown for the wild type version of MIK2 (Fig. 1). After immunoprecipitation with an anti-HA antibody, RKS1<sup>D191A</sup>-c-myc was detected when incubated with MIK2-KD<sup>D905A</sup>-HA (Fig. 1B), indicating that the aspartic acid residue of the HRD motif of MIK2 is not required for the RKS1-MIK2 interaction. Furthermore, incubation of MIK2 and MIK2<sup>D905A</sup> protein with RKS1 or RKS1<sup>D191A</sup> did not reveal MIK2 or RKS1 phosphorylation activities in vitro (Fig. 4A). Second, after introduction of MIK2<sup>D905A</sup> into the *mik2-1* mutant for a complementation test, three transgenic lines were characterized and inoculated with Xcc568. The three complemented lines showed significant phenotypic differences from Col-0, but similar phenotypes to the *mik2-1* mutant (Fig. 4B and C). MIK2<sup>D905A</sup> version was therefore not able to restore resistance in the *mik2-1* mutant at a similar level as the wild type. These results indicate that the catalytic activity of MIK2 is required to confer QDR to Xcc568. Finally, transgenic lines overexpressing MIK2 or MIK2<sup>D905A</sup> in Col-0 were also characterized (Fig. 4D). MIK2 expression was increased in 35S::MIK2 lines (517% and 161% in the two lines compared to Col-0) and reduced in 35S::MIK2<sup>D905A</sup> lines (26% and 42% in the two lines compared to Col-0) probably because native MIK2 was silenced in these lines (Supplementary Fig. S4). Inoculation tests of these lines revealed that MIK2 and MIK2<sup>D905A</sup> mis-expression in Col-0 did not significantly affect resistance to Xcc568, with the exception of one line, Col-0 (35S::MIK2) #2, which a slight reduction of resistance to Xcc568 was observed (Fig. 4D).

## MIK2 and RKS1 take place in an RK network

We demonstrated that MIK2 and RKS1 form a complex that is involved in QDR to Xcc. It is now tempting to speculate, given (i) our results showing that these two proteins share only a part of their downstream signaling pathways, and (ii) the RKS1 dependent network previously identified (Delplace et al. 2020) that these two proteins do not act isolated and require other perception/signaling proteins to orchestrate QDR. To test this hypothesis, we first identified from the literature, proteins physically interacting with MIK2 (demonstrated or putative interactions, Dataset 1). A MIK2-centralized network was reconstructed including 25 RKs, BSK3, and RKS1. Nine and five RKs interacting with MIK2 were described in the literature to be involved respectively in plant immunity (Kemmerling et al. 2007; Blaum et al. 2014; Mata-Pérez et al. 2015; Liu et al. 2016; Yeh et al. 2016; Mendy et al. 2017; van der Burgh et al. 2019; Chan et al. 2020; Laohavisit et al. 2020) and plant development (Takeuchi and Higashiyama, 2016; Wang et al. 2016; Duckney et al. 2017; Crook et al. 2020). Interestingly, 7 proteins from the RKS1 dependent protein-protein network were found highly connected to the MIK2-centralized network (Fig. 5A). Given that (i) proteins with an extracellular domain (ECD) length up to 400 amino acids may act as putative co-receptors, and (ii) proteins with a larger ECD may act as putative ligand recognition receptors

(Xi et al. 2019), this network is composed of 25 RKs described with a structure indicative of ligand binding perception proteins (including MIK2) and 12 co-receptors possibly implicated in signal transduction events (Fig. 5B).

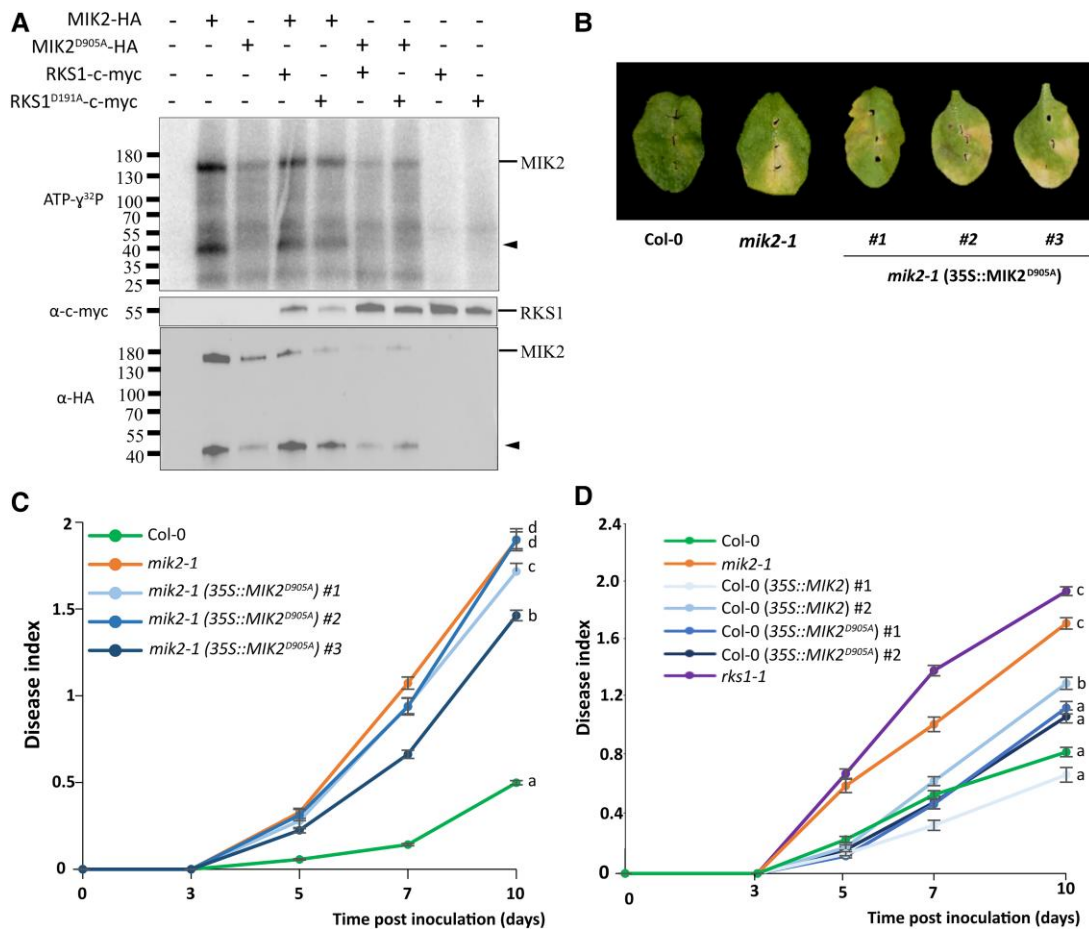
To evaluate the possible implication of some of the MIK2-RKS1 network components in QDR to Xcc, 17 mutants (corresponding to 16 genes) were collected and characterized for their response to Xcc568. The phenotypic data are summarized in Fig. 5C to E. Eight mutants corresponding to seven receptors showed a strong alteration in their response to Xcc568 (at least 30% of increased susceptibility), compared to Col-0, while mutants corresponding to seven other receptors showed a weak alteration (Fig. 5D and E) (Supplementary Tables S2 and S3). Interestingly, mutants showing a strong alteration correspond to network components known to operate in plant immunity, while the mutants with a weak effect correspond to components associated with plant development or without known function. Because MIK2 was recently proposed to operate by sensing cell wall perturbations (Van der Does et al. 2017), we also phenotyped several cell wall integrity altered mutants, i.e. *herk1*, *the1-4*, and *FER* (Cheung and Wu 2011; Nissen et al. 2016), in response to inoculation with Xcc (Supplementary Fig. S7). The *herk1-1* and *the1-4* mutants did not show a significantly altered response to Xcc568, while *fer5* mutants were slightly more resistant than Col-0. Our data suggest that the genes *HERK* and *THE1* are not significantly involved in QDR to Xcc. In addition, mutants for MIK2-like, the closest homolog of MIK2 (60% AA identity), and for MIK1, a receptor also interacting with the Male Discoverer 1 receptor, show only weak alteration in response to Xcc (Supplementary Fig. S7). Taken together, these results indicate that at least seven RKs, highly connected to MIK2 and/or RKS1, have a partial effect in QDR to Xcc568. These findings reveal the complexity of the molecular mechanisms involved in MIK2/RKS1-dependent signaling pathways, in good agreement with the genetic and molecular complexity of QDR.

## Discussion

Even if QDR is the most prevalent form of resistance in natural populations and crop fields, the identification of genes involved in the molecular mechanisms underlying QDR is still in its early stages (Roux et al. 2014b; French et al. 2016). In this study, we have identified the LRR receptor kinase MIK2 as an interactor of RKS1, a major determinant of QDR to the bacterial pathogen Xcc (Huard-Chauveau et al. 2013; Delplace et al. 2020). In addition, we found that MIK2 plays a role in QDR to Xcc, depending on its kinase catalytic activity. Taking into account (i) the RKS1-dependent network previously identified (Delplace et al. 2020), and (ii) the ability of MIK2 to interact with diverse RKs (receptors and co-receptors, Xi et al. 2019), themselves involved in interaction with RKS1 protein partners, our results suggest a role for the complex MIK2-RKS1 as a scaffolding hub in a RK network to modulate Xanthomonas determinant perception for QDR signaling and activation. The functional relevance of this RK network, centered on the MIK2-RKS1 complex, has been addressed by mutational analysis, thereby revealing the involvement of several RK components in QDR regulation. While the molecular mechanisms underlying QDR remain poorly characterized, combining functional validation, protein-protein interaction analysis, and network reconstruction, revealed insights into a perception system that regulates QDR in response to Xcc.

## The cell surface receptor-like kinase MIK2 interacts with RKS1 to activate QDR

By using the yeast two-hybrid system, we previously identified 43 proteins as putative interactors of RKS1, including 21 metabolism-

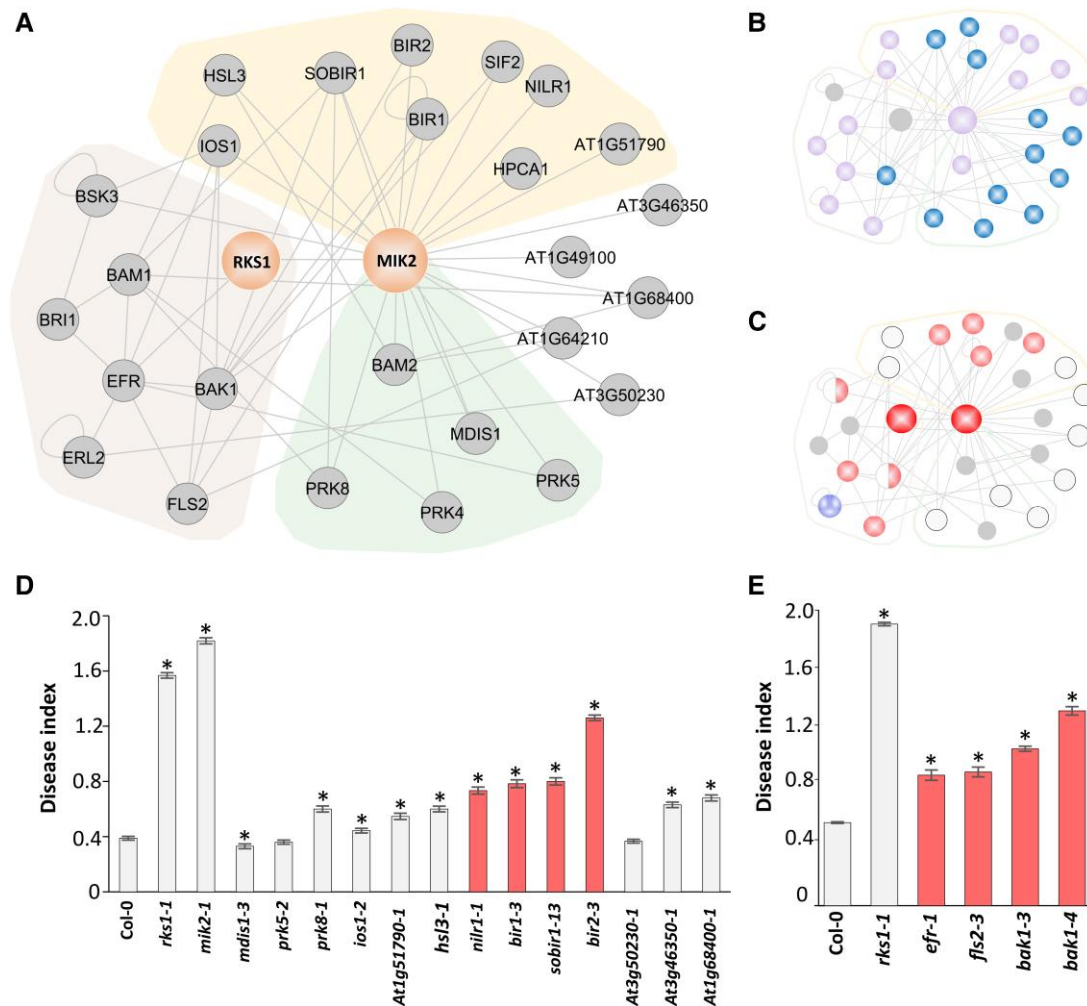


**Figure 4.** The catalytic activity of MIK2 is required for resistance to *Xcc568* but overexpression of MIK2 and MIK2<sup>D905A</sup> in Col-0 does not increase resistance to *Xcc568*. **A**) Kinase assay with protein extracts from *N. benthamiana* leaves transformed by *Agrobacterium* carrying the constructs MIK2-HA, MIK2<sup>D905A</sup>-HA, RKS1-c-myc, or RKS1<sup>D191A</sup>-c-myc. The total protein extracts were subjected to anti-HA or anti-c-myc immunoprecipitation. The immunoprecipitated proteins were incubated for 2 h at room temperature with ATP- $\gamma$ <sup>32</sup>P. The proteins were separated on a 4%–15% SDS-PAGE gel and detected by anti-HA, anti-c-myc immunoblot, and the presence of ATP- $\gamma$ <sup>32</sup>P by autoradiography. Black triangles indicate the potential MIK2 kinase domain, probably cleaved due to the protein extraction procedure. **B**) Disease symptoms were observed on leaves of *mik2-1* mutant, three *mik2-1* complemented lines with a 35S::MIK2<sup>D905A</sup> construct and wild-type plants, 10 days postinoculation with a bacterial suspension of *Xcc568* adjusted to  $2.10^8$  cfu/mL. Images were digitally extracted for comparison. The *mik2-1* image is the same as the one in Fig. 2B. **C**) Time course evaluation of the disease index of lines overexpressing MIK2 or MIK2<sup>D905A</sup> in Col-0 (35S promoter construction), *rks1-1* and *mik2-1* mutants and the wild type plants inoculated with a bacterial suspension of *Xcc568* adjusted to  $2.10^8$  cfu/mL. Means and SEM were calculated from 5 plants per line and based on 3 or 4 independent experiments (60 to 80 values by point). Statistical tests were performed by comparing the disease index kinetics modeling differences, with a P-value threshold of 0.05. a, b, c, or d represent statistical groups based on comparison of disease index kinetics. **D**) Time course evaluation of the disease index of lines overexpressing MIK2 or MIK2<sup>D905A</sup> in Col-0 (35S promoter construction), *rks1-1* and *mik2-1* mutants and the wild type plants inoculated with a bacterial suspension of *Xcc568* adjusted to  $2.10^8$  cfu/mL. Means and SEM were calculated from 5 plants per line and based on 3 independent experiments (60 values by point). a, b, or c represent statistical groups based on comparison of disease index kinetics performed as in **D**).

related proteins and 6 signaling-related components (ex: EFR, KIN11, SGT1a, MKP1, RANBP1, and NLP7) (Delplace et al. 2020). Here, we demonstrated the interaction between RKS1 and the kinase domain of the receptor-like kinase MIK2. Co-localization and BiFC experiments confirmed the subcellular localization of the two proteins and their interaction in the plasma membrane. Interestingly, RKS1 is localized in the plasma membrane, cytoplasmic tracks, and the nucleus (Delplace et al. 2020), suggesting different functions for RKS1 including a contribution to pathogen perception events. The NLR ZAR1 was demonstrated to form a complex with RKS1 in the cytoplasm to perceive the injected effector AvrAC from *X. campestris* (Adachi et al. 2019; Wang et al. 2019). The interaction of RKS1 with a receptor-like protein such as MIK2, suggests that RKS1 might also be involved in the extracellular perception of pathogen determinants or endogenous peptides at the plasma membrane. Based on our findings, RKS1 might be part of

membrane complexes that, similarly to MIK2, include cell surface receptors required for transduction events (Antolín-Llovera et al. 2012). RKS1-receptor complexes may be dynamic, depending on the nature of the pathogen. Similar to the pseudokinase domain of the receptor BIR2 involved in the allosteric regulation of the receptor BAK1 (Blau et al. 2014), the RKS1-MIK2 complex, MIK2 or RKS1, could act as a scaffolding protein complex and participate in the regulation of signaling pathways as a molecular switch, depending on the context of protein-protein interactions (Roux et al. 2014a; Murphy et al. 2017). Interestingly, MIK2 seems connected with multiple sensing systems including cell wall integrity sensing (CWIS) (Rhodes et al. 2021), root growth and response to abiotic and biotic stresses (Julkowska et al. 2016; Van der Does et al. 2017). In particular, MIK2 is involved in the perception of the phytoytokine peptide SCOOP12, by heterodimerizing with BAK1 (Rhodes et al. 2021). While no plant developmental function has





**Figure 5.** MIK2 and RKS1 as hubs of an RK network. **A)** MIK2 protein–protein interaction subnetwork was reconstructed from STRING and BioGrid databases and plotted with Cytoscape. The light brown area indicates proteins recovered from the RKS1 dependent network (Delplace et al. 2020). The yellow area indicates RKs interacting with MIK2 and described to play a role in plant immunity (Kemmerling et al. 2007; Blaum et al. 2014; Mata-Pérez et al. 2015; Liu et al. 2016; Yeh et al. 2016; Mendy et al. 2017; van der Burgh et al. 2019; Chan et al. 2020; Laohavisit et al. 2020). The green area indicates RKs interacting with MIK2 and described to be implicated in plant development (Takeuchi and Higashiyama, 2016; Wang et al. 2016; Duckney et al. 2017; Crook et al. 2020), the uncolored zone corresponds to hypothetical RKs. The gray lines between nodes indicate protein–protein interaction. **B)** In the MIK2 protein–protein interaction network, purple circles represent RKs from the “ligand perceiving group” (Xi et al. 2019), blue circles represent LRR-RKs from the “co-receptor group” and gray circles, other proteins. The gray lines between nodes indicate protein–protein interaction. The colored outlines represent function of proteins (light brown, proteins recovered from the RKS1 dependent network; yellow, RKs interacting with MIK2 and described to play a role in plant immunity; green, RKs interacting with MIK2 and described to be implicated in plant development). **C)** Each circle represents the phenotype of the mutant corresponding to the protein component of the network. Colored circles indicate some mutants significantly affected in their response to *Xcc568*. Mutants represented in red are significantly affected in their response to *Xcc* (at least 30% of increased susceptibility compared to Col-0 disease index), according to **D)** and **E)**, and those represented in purple are more resistant compared to Col-0 according to Delplace et al. (2020). The circle is divided according to the number of tested mutants. White circles correspond to mutants with a phenotype similar to the one of the wild type line. No mutant was tested for genes represented in gray. Data presented for *ERL2* and *BSK3* were recovered from Delplace et al. (2020). The gray lines between nodes indicate protein–protein interaction. The colored outlines represent function of proteins (light brown, proteins recovered from the RKS1 dependent network; yellow, RKs interacting with MIK2 and described to play a role in plant immunity; green, RKs interacting with MIK2 and described to be implicated in plant development). **D)** Disease index of mutants corresponding to genes belonging to the MIK2-RKS1 network, at 10 dpi after inoculation with a bacterial suspension adjusted to  $2.10^8$  cfu/mL. \*Represents kinetic modeling difference with Col-0 time course, based on five independent experiments with 5 plants/mutant. Light gray bars correspond to mutants for RKs described in part **A)** and dark gray bars correspond to *rks1-1* mutant and Col-0 wild type used as controls. Mutants represented in red are significantly affected in their response to *Xcc* (at least 30% of increased susceptibility compared to Col-0 disease index). Statistical tests were performed by comparing the disease index kinetics modeling differences, with a P-value threshold of 0.05. Error bar represent SEM. **E)** Disease index at 10 dpi after inoculation in the same conditions as **D)**. \*Represents kinetic modeling difference with Col-0 time course in 3 to 5 experiments with 5 plants/mutants. Light gray bars indicate mutants for RKs from the RKS1 dependent network and dark gray bars correspond to *rks1-1* mutant and Col-0 wild type used as controls. Mutants represented in red are significantly affected in their response to *Xcc* (at least 30% of increased susceptibility compared to Col-0 disease index). Statistical tests were performed by comparing the disease index kinetics modeling differences, with a P-value threshold of 0.05. Error bar represent SEM.

been suggested for RKS1 yet, it is a major determinant of QDR for which CWIS might be a component (Zuo et al. 2015; Bani et al. 2018). To test this hypothesis, mutants for CWIS (*herk1-1*, *the1-4*, and *fer-5*) were phenotyped in response to *Xcc*. None of the CWIS

mutants was found more susceptible to *Xcc568* (Supplementary Fig. S7), suggesting that CWIS is not required to confer QDR to *Xcc*. These results show that CWIS does not generally interfere with QDR to *Xcc*, in opposition to a previous study proposing

that *MIK2* and *THE1* could function in the same pathways but distinct from the *FER* pathways, against *Fusarium oxysporum* (Coleman et al. 2020). This discrepancy might be explained by the fact that *F. oxysporum* is more of a root and cell wall invasive pathogen in comparison with the leaf vascular pathogen *Xcc* (Kubicek et al. 2014; An et al. 2020). *MIK2* may participate in the detection of multiple ligands (Wang et al. 2016; Rhodes et al. 2021), that could require *MIK2* directly, or *MIK2* interacting with other ligand-perceiving receptors (Fig. 5B). In the future, to decipher the molecular dialogues between *Xcc* and host plants, a challenge would be to identify putative *Xcc* or/and plant cell damages derived ligands perceived by *MIK2*. To initiate this investigation into potential ligands perceived by *MIK2* during *Xcc568* infection, we first examined the expression patterns of PROSCOOPs at early time points postinfection using RNA-seq data from Delplace et al. (2020). Indeed, as already mentioned, *MIK2* has been found to be involved in the perception of the phyto cytokine peptide SCOOP12, and PROSCOOP genes belong to a family encoding secreted propeptides, which are matured in SCOOP peptides (Rhodes et al. 2021; Yang et al. 2023). Interestingly, 9 PROSCOOPs were found to share the same expression pattern as *MIK2* during infection, including a strong induction of PROSCOOP12, and, in total, at least 28 PROSCOOPs are induced between 0 and 6 h after *Xcc568* infection (Supplementary Fig. S8). Second, we identified a SCOOP-like motif in the XopAM protein from *Xcc568* (Supplementary Fig. S9), a putative *MIK2* ligand that constitutes an interesting target for future work. Unlike other AvrE-T3Es, XopAM may function as a lipase in *N. benthamiana*, affecting membrane integrity and inducing cell death (Xie et al. 2023), potentially releasing XopAM into the apoplast where the SCOOP-like motif could be perceived by *MIK2*. However, the function of XopAM in the *Xcc*–plant interaction remains unclear. XopAM contains a translocation signal (Potnis et al. 2011) but is not located near the T3SS cluster, and type III secretion chaperone genes are not found adjacent to the gene encoding XopAM (Degrave et al. 2015). Thus, despite being part of the core type III secretome in multiple *Xcc* strains (Guy et al. 2013), its role as a T3E effector is unconfirmed, and its translocation via T3SS needs to be experimentally confirmed. In conclusion, endogenous SCOOPs and/or pathogen SCOOP-like peptide mimics, might play a critical role in the early perception of *Xcc* by *MIK2* or *MIK2* in association with other immune components, and represent promising putative actors of this interaction, similarly to nematode-encoded RALF peptide mimics reported to facilitate parasitism for example (Zhang et al. 2020). Approaches including physical protein–ligand interactions using *MIK2* and other potential RKs identified in this study would help to shed some light on the perception events responsible for QDR. To conclude, *MIK2* with its diverse functions in CWIS and responses to abiotic and biotic stress could play different functional roles depending on its interacting partners. As a complex with *RKS1*, it appears to be a major player in plant immune responses to the bacterial pathogen *Xcc*.

### RKS1–MIK2 as a regulatory complex for QDR signaling?

While receptor kinases, and among them the LRR-receptor kinases, constitute the largest protein kinase family in plants, a large part of these receptors and their putative interactors are still functionally uncharacterized. Here, we showed that *MIK2* confers QDR to *Xcc568* at a similar level as *RKS1*. Analysis of the double mutant *rks1-1/mik2-1* revealed that *MIK2* and *RKS1* probably act in a common pathway in response to *Xcc*, which might represent only a

part of the immune response, as the double mutant express an intermediary phenotype, more susceptible than the resistant reference accession, less susceptible than the susceptible reference accession, but slightly more susceptible than the single mutants. To further explore their respective functions, we measured the expression of a set of 36 genes from the previously identified *RKS1*-dependent network (Delplace et al. 2020) or described to be dependent on *MIK2* (Hou et al. 2021). A major part of these genes (44%) showed an expression dependent on the mutation *mik2*. For this set of genes, 31% (including *RGLG2* and *VAMP724*, presented in Fig. 3) showed an expression profile dependent on both mutations *rks1* and *mik2*, confirming the existence of common pathways activated by both proteins. We observed that the reduced gene expression of *RGLG2* and *VAMP724* in *rks1-1* and *mik2-1*, which was rescued in *rks1-1/mik2-1*, suggesting a potential epistatic interaction between *MIK2* and *RKS1*, where one mutation restores expression affected by the second mutant. *RGLG2* was described as a RING domain ubiquitin E3 ligase implicated in stress drought adaptation and *VAMP724* as SNAREs protein involved vesicle-associated trafficking (Yu et al. 2020; He et al. 2023). Eleven genes were found specifically dependent of *MIK2*, such as the transcription factor *WRKY30*, implicated in abiotic stress tolerance (Scarpecci et al. 2013). These different roles can be explained by the putative interactions of *MIK2* with other signaling partners in plasma membrane receptor complexes (He et al. 2018). Together these data confirm the implication of *MIK2* and *RKS1* in QDR signaling, potentially via the *MIK2* kinase domain. Indeed, we showed that *MIK2*, as classical RD (arginine and aspartic acid) RKs such as *BAK1* and *CERK1* (Oh et al. 2010; Suzuki et al. 2018), exhibits an autophosphorylation activity dependent on the aspartic acid 905 residue. Similar to *BRI1* that is maintained in an inactive stage by autophosphorylation (Oh et al. 2012), *MIK2* phosphorylation activity could be required for autoregulation. Surprisingly, the *MIK2* aspartic acid 905 residue appeared not essential for *RKS1* interaction. These results suggest, in agreement with the functioning of multiple plasma membrane receptor complexes (Burkart and Stahl 2017) that *RKS1* might act as a scaffold for receptor complex assembly in the context of QDR. A similar role was described for the PTI receptors *FLS2* and *EFR*, with the *FER* scaffolding receptor (Stegmann et al. 2017). Our results are in good agreement with the recent “Invasion model” proposing that there might be no clear distinction between PTI, ETI, and more widely the different forms of plant immunity responses (Cook et al. 2015; Kanyuka and Rudd 2019) (including QDR), and that broad-spectrum immunity might depend on complex networks of cell surface immune receptors in most cases. Here, we extend this model by proposing that *RKS1* participates in surface and intracellular perception mechanisms (in interaction either with *MIK2* or *ZAR1*), which are initiated either in the apoplast or the cytosol.

### MIK2/RKS1 complex as a hub to coordinate an RK perception network?

LRR-RKs sense a wide array of molecules produced exogenously or endogenously and regulate plant growth and immunity. They can operate in a regulatory network (Smakowska-Luzan et al. 2018) where small LRR-RKs (co-receptors) are involved (i) in fine-tuning (by activation or stabilization) of ligand binding receptors, and (ii) as regulatory scaffolds for the organization of the signaling network (Xi et al. 2019). In this context, understanding the functioning of the *MIK2/RKS1* complex in QDR needs to be replaced within a protein–protein interaction (PPI) network. From the literature,

we identified MIK2 interacting proteins, and generated a network including, beyond RKS1, RKs from the RKS1 dependent network. MIK2, a ligand binding receptor, takes place in a highly connected RK network including signaling proteins involved either in immunity or plant development (Fig. 5). Interestingly, the MIK2-RKS1 network reveals no clear co-receptor hub (linked to ligand-perceiving receptors) such as BAK1 (Smakowska-Luzan et al. 2018). In addition, MIK2 is connected to diverse receptors and not only with co-receptors, such as the ligand-perceiving receptor BAM1 that forms a complex with several co-receptors of the CIK family (Cui et al. 2018). The MIK2-RKS1 centralized network appears more diverse than co-receptor and ligand perceiving receptor associations (Xi et al. 2019). This might be due to the dynamic complexity of the perception events involved in QDR, and probably also to the insufficient characterization or absence of some LRR-RKs (or other receptors) in this reconstructed network. A functional analysis of this network by mutant analysis showed that seven RKs are involved in QDR to Xcc with a strong effect (Fig. 5), described to be related to plant immunity and connected with MIK2 (BIR1, BIR2, NIRL1, SOBIR, EFR, FLS2, and BAK1) (Kemmerling et al. 2007; Blaum et al. 2014; Liu et al. 2016; Laohavisit et al. 2020; Rhodes et al. 2021). Among these RKs, the MIK2/RKS1 complex might recruit some RKs as a perception system to trigger QDR, similarly to the co-receptor BAK1. Interestingly, BIR2 plays a major positive regulatory role in QDR to Xcc (Fig. 5). BIR2 has previously been reported as a negative regulator of PTI in Arabidopsis (Halter et al. 2014a). Interaction with BAK1 in a kinase activity-dependent manner negatively controls the formation of complexes between BAK1 and specific ligand-binding receptors, to regulate plant immune responses (Halter et al. 2014b). More recently and in line with our findings, BIR2 has been found to positively regulate resistance to bacterial and oomycete pathogens in *N. benthamiana* (Liu et al. 2023), emphasizing a key role for this RK in immunity. Beyond BIR2, multiple RKs contribute quantitatively to resistance to Xcc, and the association of RKS1 with the kinase domain of different RKs suggests that RKS1 may act as a receptor platform regulating the formation of RK complexes with specific ligand-binding receptors. In this context, MIK2 plays a central role with RKS1, by perceiving multiple peptides/ligands (Wang et al. 2016; Rhodes et al. 2021) and by recruiting a diversity of co-receptors. The perception by RKs of multiple signals (MAMPs/DAMPs or effectors released during infection) could trigger diverse interconnected signaling pathways, where MIK2/RKS1 complex may potentiate different pathways, depending on the nature of the ligand perceived (Ngou et al. 2021). Thus, seven other RKs connected with MIK2, including co-receptors with unknown functions (AT1G68400 and AT1G64210) or with plant developmental functions (MDIS1 and PERK8) are implicated in QDR to Xcc but to a much lesser extent, indicating that MIK2 in association with RKS1, could mainly recruit RKs involved in immune responses. Similar to the receptor BAK1 described to be a co-receptor for multiple LRR receptors involved in PTI and plant development (Postel et al. 2010; Zhou et al. 2019), MIK2 might require specific RKs or other signaling components to coordinate immunity and development perception systems. Interestingly, cytoplasmic kinases are also known as molecular switches between plant development and immunity pathways, such as BSKs (brassinosteroid kinases) (Lin et al. 2013). BSK3, which is implicated in brassinosteroid signaling, confers partial resistance to Xcc and thus can be a key regulatory compound in the MIK2/RKS1-centralized network (Sreeramulu et al. 2013; Delplace et al. 2020). In this line, BSK3 was also described as a scaffolding protein for plant development (Ren et al. 2019).

Another interesting feature of this network is the presence of four RKs from the RKS1-dependent network: ERL2, BAK1, EFR, and FLS2. EFR and FLS2 are known to directly perceive pathogen determinants (Chinchilla et al. 2006; Zipfel et al. 2006) and can therefore participate in the perception of multiple pathogen determinants, which is in good agreement with the view of QDR as a complex network integrating diverse pathways in response to multiple pathogenic determinants (Roux et al. 2014b). While receptors could participate directly or indirectly to pathogen perception with strong implication in QDR to Xcc, such as BAK1 or BIR2, other receptors with a weak implication in QDR, like MDIS1 or IOS1, could have either redundant functions with other receptors or only an indirect implication in Xcc perception. In the future, consolidation of the PPI network, investigation of other possible interactions among the different components and testing whether the contribution of these RKs in resistance to Xcc depends on the presence of RKS1 or MIK2, will permit to confirm and extend the complexity of this network. Taken together, our results suggest that the RKS1/MIK2 complex in relation to a number of additional RKs might participate in a perception system and act as a scaffold hub to coordinate the assembly of multiple perception complexes.

In summary, the MIK2/RKS1 complex takes place in an RK network in the plasma membrane and might be involved in the perception of multiple pathogen ligands, subsequently leading to QDR, each component participating partially to QDR to Xcc. This work constitutes a strong basis for deciphering molecular mechanisms involved in the perception systems related to QDR.

## Materials and methods

### Plant material

Arabidopsis plants were grown on Jiffy pots under controlled conditions (Lacomme and Roby 1996), in a growth chamber at 22 °C with a 9-h photoperiod at 192  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . We used the wild-type line Columbia (Col-0) (Wilson et al. 2001; Li et al. 2006) and the following mutant lines (all in Col-0 background): *rks1-1* (Huard-Chauveau et al. 2013), other mutants from the GABI-kat (<http://www.gabi-kat.de>) or SALK (<http://signal.salk.edu>) seed libraries and the susceptible accession to Xcc568 Kas-1 (Huard-Chauveau et al. 2013). For each mutant line, the T-DNA insertion was determined by sequencing of the T-DNA borders (GABI o8409 or LBb1.3\_SALK primer) and of the flanking regions (Supplementary Table S4). The double mutant *mik2-1/rks1-1* was obtained by crossing *mik2-1* pistils with *rks1-1* pollen.

For transient expression assays, *N. benthamiana* plants were cultivated 4-week at 21 °C and under 15 h light period/9 h dark period.

### Bacterial material

*Agrobacterium tumefaciens* strains GV3101, GV3103, and C58C1 were grown at 28 °C on YEB medium with 50  $\mu\text{g.mL}^{-1}$  rifampicin, complemented with 10  $\mu\text{g.mL}^{-1}$  kanamycin and 10  $\mu\text{g.mL}^{-1}$  gentamicin (GV3101), with 100  $\mu\text{g.mL}^{-1}$  carbenicillin and 10  $\mu\text{g.mL}^{-1}$  gentamicin (GV3103) or tetracyclin (C58C1). For transient expression assays for fluorescence microscopy, the *Agrobacterium* strain GV3101 carrying the corresponding mRFP1 and eGFP constructs was used. Arabidopsis seedlings were transformed according to Marion et al. (2008). Overnight cultures of *A. tumefaciens* were resuspended in 5% sucrose supplemented with acetosyringone (200  $\mu\text{M}$ ). Then, 1 week-old seedling were vacuum-infiltrated with the *Agrobacterium* solution. For co-immunoprecipitation

experiments, *Agrobacterium* strain C58C1 carrying the corresponding HA and c-myc constructs was prepared as GV3101 strain. *N. benthamiana* plants were infiltrated and leaf disks harvested 28 h after inoculation.

Xcc inoculation tests were done with the strain LMG568/ATCC33913 (Xcc568) (Da Silva et al. 2002). Cultures of Xcc568 were grown at 28 °C on Kado medium (Kado and Heskett 1970) supplemented with 50 µg.mL<sup>-1</sup> rifampicin and 25 µg.mL<sup>-1</sup> kanamycin.

## Constructs and plant transformation

The MIK2 full length constructs were performed by amplification of the MIK2 (AT4G08850) cDNA coding sequence using, respectively, [attb1\_MIK2\_full\_length and attb4\_MIK2\_NO\_STOP] as primers (Supplementary Table S5). The catalytic MIK2<sup>D905A</sup> mutants were generated using, respectively, [MIK2\_D905A\_fw and MIK2\_D905A\_rev] primers (Supplementary Table S5). MIK2-KinaseDomain was amplified with [attb1\_MIK2\_KD\_fw and attb4\_MIK2\_NO\_STOP] primers (Supplementary Table S5). PCR products were cloned into the multisite Gateway entry vector pBS-DONR P1-P4. 3xHA, 5xc-myc, mRFP1, and eGFP tags were cloned into the entry vector pBS-DONR P4-P2. To fuse MIK2 constructs with 3xHA, 5xc-myc, mRFP1, or eGFP tags, both vectors were mixed with the 35Sp plant expression vector pEarleyGate100 (Lema Asqui et al. 2018) and recombined with LR clonase II (Invitrogen) described previously (Gu and Innes 2011). We used RKS1<sup>D191A</sup> in pBS-DONR P1-P4 described in Delplace et al. (2020) fused as MIK2 with c-myc in pEG100. The plasmid constructs with the different tags fused to the genes of interest are reported in the Supplementary Table S6.

The MIK2-OE/Col-0 and MIK2-OE/mik2-1 constructs were introduced in the C58C1 *Agrobacterium* strain for transformation of Arabidopsis Col-0 (Clough and Bent 1998). Selection of transformant plants was performed by spraying glufosinate ammonium (BASTA) at 10 mg.L<sup>-1</sup> on soil-grown plants. Harvested seeds were spread on MS medium containing 50 µM of phosphinothricin for selection of homozygous transgenic plants.

## Plant phenotyping and statistical analyses

The disease symptoms of mutant lines were evaluated after inoculation with a bacterial suspension adjusted to 2.10<sup>8</sup> cfu.mL<sup>-1</sup>, in 3 independent experiments (Lacomme and Roby 1996), as compared to Col-0 and *rks1-1*. Four leaves per plant and four 28-day old plants per line were inoculated by piercing and scored as previously described (Meyer et al. 2005), at 0, 3, 5, 7, and 10 days postinoculation.

We fitted the temporal relation of disease index between the tested mutant line and Col-0 by a second order polynomial using the *lm* library under the R environment (<https://www.R-project.org/>). Kinetics of disease index was considered similar if the coefficient of the second order was not significantly different from 0 and if the slope was not significantly different from 1. P-value numbers represent kinetic modeling deference with Col-0, 0 = P > 0.05 and 1 = P ≤ 0.05.

## RNA isolation and RT-qPCR

RNA extraction was performed with NucleoSpin RNA plus kit (Macherey-Nagel). RNA extraction was performed with leaves from 28-days-old healthy plants (6 plants and 3 leaves/plant) or inoculated with the Xcc568 (6 h postinoculation). RT-qPCR analysis was performed as described by Khafif et al. (2017). The gene AT2G28390 (MONENSIN SENSITIVITY 1) was used as an internal control as it is known to be stable in our physiological conditions (Czechowski et al. 2005). Average ΔCp was calculated from three

experiments and data were expressed as fold induction for each point as compared to the wild type. Primers have been designed via the Roche website (<http://www.lifescience.roche.com>) (Supplementary Tables S7 and S8). Results were analyzed using the LC480 on-board software, release version 1.5.0.39. Statistical results were generated with a GLM procedure under the R environment. A correction for the number of tests was performed to control the false discovery rate (FDR) at a nominal level of 5%.

## Co-immunoprecipitation assays

Total proteins were extracted from *N. benthamiana* leaves harvested 28 h after treatment (transient expression experiments) using the extraction buffer [10% glycerol, 50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM DTT, 0.05% Nonidet P-40, cOmplete Mini EDTA-Free protease inhibitor cocktail (Sigma), PhosSTOP EASYpack phosphatase inhibitors (Roche) and a spatula tip of polyvinylpyrrolidone]. The samples were incubated with anti-c-myc or anti-HA magnetic beads (Thermo Scientific) for 2–4 h and washed with the same buffer without phosphatase inhibitors. Proteins were then separated on SDS-PAGE 4%–15%. After incubation with rat anti-HA:HRP (3F10 clone, Roche [dilution 1:3000]) or mouse anti-Myc:HRP antibodies (9E10 clone, Roche [dilution 1:3000]), proteins were visualized using the Bio-Rad Clarity Western ECL kit and the ChemiDoc Imaging System (Biorad).

## Fluorescence microscopy

Fluorescence images were acquired using a Leica SP8 confocal microscope equipped with a water immersion objective lens (25×, numerical aperture 1.20; PL APO) (Imaging TRI-Genotoul plate-form). GFP and RFP fluorescence was excited with the 488 nm ray line of the argon laser (10% intensity) or the 552 nm ray line of the He-Ne laser (10% intensity) respectively. The emission recording bands were set in the 505 to 530 nm range for GFP detection, hybrid detector at 100%, and 580 and 620 nm range for RFP detection, with a PMT gain of 775 V. Image acquisition was done in the sequential mode using Leica LCS software and analyzed using the ImageJ software. Representative confocal images are shown after histogram normalization.

## Bimolecular fluorescence complementation

The coding sequences of RSK1, MIK2, and ZAR1 were amplified by PCR from Arabidopsis Col-0 cDNA using the primers indicated in Supplementary Table S4. To generate C-terminal fusion proteins with the C-terminal or N-terminal fragment of YFP, the genes were cloned into the expression vectors pEG100 (Earley et al. 2006) using the Gateway technology (Invitrogen) (Gu and Innes 2011). The constructs expressing cYFP- or nYFP-tagged proteins in pEG100 were co-transfected into Arabidopsis Col-0 mesophyll protoplasts according to the previously described protocol (Yoo et al. 2007) and co-transfected in *Nicotiana benthamiana* leaves using GV3101 *Agrobacterium* strain or GV3103 *Agrobacterium* strain for MtLRR1I-cYFP and MtLYK3-cYFP constructions (Lefebvre et al. 2010). The fluorescence was analyzed 16 h (Arabidopsis) or 36 h (*N. benthamiana*) after transfection by confocal laser microscopy (Leica, SP8). YFP was excited with the 514 nm ray line of the argon laser, at 5% of its nominal power. The emission detection range was set between 525 and 575 nm, with a PMT gain of 909.6 V.

## Subnetwork reconstruction

Forty-six experimentally identified interactors of MIK2 were recovered from Arabidopsis BioGRID protein interaction datasets Version 4.0.189 (Oughtred et al. 2019) and from the literature

completed with 8 putative interactors from RK network described in Xi et al. (2019). Details of protein–protein interactions are presented in Dataset 1. RK annotation “ligand-perceiving” and “co-receptor” groups were recovered from Xi et al. (2019). Plant processes for each gene were verified with the current literature. Protein–protein interactions were plotted with Cytoscape software V3.7.2.

### Accession numbers

Sequence data referenced in this study are available via the SRA database under accession number SRP233656.

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### Author contributions

D.R. designed research. F.D. and C.H.-C. performed research. F.D., C.H.-C., F.R., and D.R. analyzed data. F.D., C.H.-C., F.R., and D.R. wrote the paper.

### Supplementary data

The following materials are available in the online version of this article.

**Supplementary Figure S1.** Full-length MIK2 interacts with RKS1.

**Supplementary Figure S2.** MIK2 physically interacts with RKS1 at the plasma membrane by BiFC in Arabidopsis protoplasts.

**Supplementary Figure S3.** MIK2 physically interacts with RKS1 at the plasma membrane by BiFC in *N. benthamiana* leaves.

**Supplementary Figure S4.** MIK2 expression in mutants and other lines used in this study.

**Supplementary Figure S5.** *mik2* mutants are more susceptible to *Xcc568* infection.

**Supplementary Figure S6.** Analysis of *rks1-1*, *mik2-1* and *rks1-1/mik2-1* mutant lines in response to *Xcc568*.

**Supplementary Figure S7.** Cell wall-sensing mutants are not affected in their QDR to *Xcc568*.

**Supplementary Figure S8.** Nine endogenous PROSCOOPs, including PROSCOOP12, are differentially expressed during *Xcc568* infection, similar to the expression pattern of *MIK2*.

**Supplementary Figure S9.** A SCOOP12-like peptide found in the XopAM effector.

**Supplementary Table S1.** Gene expression quantified by RT-qPCR six hours after infiltration with *Xcc568* at  $2 \times 10^8$  cfu/mL.

**Supplementary Table S2.** Molecular analysis of insertional mutants of the RK network.

**Supplementary Table S3.** Molecular analysis of insertional mutants of the RKS1-dependent subnetwork.

**Supplementary Table S4.** List of primers used for mutant genotyping.

**Supplementary Table S5.** Primers used for *MIK2* constructs.

**Supplementary Table S6.** List of pEarleyGate100-based constructs used in this study.

**Supplementary Table S7.** List of RT-qPCR primers used for expression analysis.

**Supplementary Table S8.** List of RT-qPCR primers used for the molecular characterization of T-DNA mutants.

**Supplementary Data Set 1.** Protein–protein interactions used to generate the RKS1/MIK2 PPI network.

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*Conflict of interest statement.* None declared.

### Data availability

The data underlying this article are available in the article and in its online supplementary material.

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