NbCSPR underlies age-dependent immune responses to bacterial cold shock protein in *Nicotiana benthamiana*

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Plants use receptor kinases (RKs) and receptor-like proteins (RLPs) as pattern recognition receptors (PRRs) to sense pathogen-associated molecular patterns (PAMPs) that are typical of whole classes of microbes. After ligand perception, many leucine-rich repeat (LRR)-containing PRRs interact with the LRR-RK BRI1-ASSOCIATED KINASE 1 (BAK1). BAK1 is thus expected to interact with unknown PRRs. Here, we used BAK1 as molecular bait to identify a previously unknown LRR-RLP required for the recognition of the csp22 peptide derived from bacterial cold shock protein. We established a method to identify proteins that interact with BAK1 only after csp22 treatment. BAK1 was expressed transiently in *Nicotiana benthamiana* and immunopurified after treatment with csp22. BAK1-associated proteins were identified by mass spectrometry. We identified several proteins including known BAK1 interactors and a previously uncharacterized LRR-RLP that we termed RECEPTOR-LIKE PROTEIN REQUIRED FOR CSP22 RESPONSIVENESS (NbCSPR). This RLP associates with BAK1 upon csp22 treatment, and *NbCSPR*-silenced plants are impaired in csp22-induced defense responses. *NbCSPR* confers resistance to bacteria in an age-dependent and flagellin-induced manner. As such, it limits bacterial growth and *Agrobacterium*-mediated transformation of flowering *N. benthamiana* plants. Transgenic expression of *NbCSPR* into *Arabidopsis thaliana* conferred responsiveness to csp22 and antibacterial resistance. Our method may be used to identify LRR-type RKs and RLPs required for PAMP perception/responsiveness, even when the active purified PAMP has not been defined.

plant immunity | PAMP | receptor | BAK1 | *Nicotiana benthamiana*

Plants and animals sense microbes by detecting a range of pathogen-associated molecular patterns (PAMPs). PAMPs are recognized directly by pattern recognition receptors (PRRs) located on the cell surface. In plants, PRRs usually belong to the receptor kinase (RK) or receptor-like protein (RLP) classes and often contain leucine-rich repeat (LRR) or carbohydrate-binding LysM extracellular domains (1). Perhaps the best-studied PRR is the LRR-RK FLAGELLIN SENSING 2 (FLS2) that recognizes bacterial flagellin or its peptide derivative flg22 (2–4). FLS2 and several other LRR-type receptors require the LRR-RK BRI1-ASSOCIATED KINASE 1 (BAK1) for signal transduction. BAK1 (SERK3) is part of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family in *Arabidopsis thaliana*. BAK1 is sometimes functionally redundant with SERK4/BAK1-LIKE 1 (BKK1) (5). In many cases, BAK1 interacts with receptors in a ligand-induced manner (4–8). The BAK1-INTERACTING RKs 1 and 2 (BIR1 and BIR2) negatively regulate BAK1 (9, 10). BIR2 was identified by BAK1 pull-down and is released from the BAK1-FLS2 complex during flg22 perception, whereas BIR1 negatively regulates BAK1-mediated cell death before complex activation. The *bir1-1* cell death phenotype is rescued by a mutation in *SUPPRESSOR OF BIR1-1* (*SOBIR1*), *sobir1-1*. SOBIR1 is a LRR-RK that interacts with RLPs, including the tomato LRR-RLPs Cf-4 and Ve1 (11) and the RLPs ReMAX (12) and RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASES1 (RBPG1/RLP42) from *Arabidopsis thaliana* (13). SOBIR1 is also required for responses to an elicitor-containing fraction from the necrotrophic fungus *Sclerotinia sclerotiorum* mediated by RLP30 (14) and forms a constitutive, ligand-independent complex with RLP23 in *A. thaliana*, which recruits BAK1 upon perception of the PAMP NECROSIS AND ETHYLENE-INDUCING PEPTIDE 1-LIKE PROTEIN 20 (nlp20) (15). *Nicotiana benthamiana* contains two SOBIR1 homologs, NbSOBIR1 and NbSOBIR1-like (11).

Activation of PRRs leads to PAMP-triggered immunity (PTI) (16). PTI is associated with cellular phenomena such as extracellular alkalinization, influx of apoplastic Ca^{2+}, production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), and reprogramming of host gene expression (17). Adapted bacterial pathogens evade PTI by altering PAMPs to avoid recognition or by secreting virulence effector proteins into the host cytoplasm to inhibit PTI (18). Reduced PTI is associated with disease (18), but is also essential for *Agrobacterium*-mediated plant transformation and interactions with symbiotic bacteria (19). Bacteria that are not recognized by FLS2 elicit PTI through the perception of alternative PAMPs, and several PAMPs are recognized only by certain plant families (20). For example, *A. thaliana* recognizes the bacterial PAMP elongation factor-Tu through the LRR-RK ELONGATION FACTOR-TU RECEPTOR (EFR) (21). EFR recruits BAK1 after perception of the EF-Tu–derived peptide elf18, illustrating the capacity of BAK1 to interact with different receptors (8). Likewise, the cold shock protein (CSP) was

Significance

Plants detect pathogens by surface-localized receptors. Few such receptors are known. The coreceptor BRI1-ASSOCIATED KINASE 1 (BAK1) is a frequent member of activated receptor complexes. The proteomics strategy described here uses BAK1 as molecular bait to identify potential receptors that are specifically activated by pathogen components. We demonstrate this approach by identifying *Nicotiana benthamiana* RECEPTOR-LIKE PROTEIN REQUIRED FOR CSP22 RESPONSIVENESS (NbCSPR). We show that NbCSPR is required for immune responses initiated by the bacterial cold shock protein, confers age-dependent immunity against bacteria, and restricts the transformation of *N. benthamiana* cells by *Agrobacterium*. Manipulation of this gene will provide new options for disease control and genetic transformation of crop species.

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identified from the bacterium *Staphylococcus aureus* as a PAMP that is perceived specifically by members of the plant family *Solanaceae* (22). CSP contains a conserved cold-shock domain (CSD), and the N-terminal 22-amino-acid sequence of the CSP consensus sequence (csp22) elicits immune responses in a BAK1-dependent manner (7, 22). However, a receptor required for CSP-mediated immunity has not yet been identified, despite identification of this PAMP over 10 y ago. Here, we describe a proteomics approach to identify RKs or RLPs required for PTI in response to csp22 using BAK1 as molecular bait. We confirm its utility by identifying a LRR-RLP required for CSP-induced PTI in *N. benthamiana* (*N. benthamiana* RLP REQUIRED FOR CSP22 RESPONSIVENESS). NbCSPR induced immune responses after csp22 treatment in an *NbBAK1*-dependent manner and restricted the growth of adapted and nonadapted bacteria. We further show that perception of CSP from *Agrobacterium tumefaciens* limits transformation of *N. benthamiana* and that interfamily transfer of NbCSPR can be a useful strategy to enhance bacterial disease resistance in non-Solanaceaeous plants.

Results

csp22 Responses Are Age-Dependent in *N. benthamiana*. Four- to 5-wk-old *N. benthamiana* plants before the onset of flowering are commonly used to measure immunity and for transient *Agrobacterium*-mediated transformation (23). Unlike flg22-induced events, csp22-dependent responses are weak and inconsistent in plants of this age. We found that csp22-induced responses were higher in flowering *N. benthamiana* plants. Under the growth conditions used here, plants were 6 wk old when they flowered. We measured PTI responses including ROS production, Ca^{2+} influx, activation of MAPKs, and up-regulation of PAMP-induced gene (PIGs) expression. All responses triggered by csp22 were greater in 6-wk- than in 4-wk-old plants, but this effect was not seen for flg22 (*SI Appendix*, Figs. S1 and S2). Therefore, plants at this developmental stage were used to identify proteins required for csp22 responsiveness in *N. benthamiana* and for all subsequent experiments unless otherwise indicated.

Identification of CSPR from *N. benthamiana* Using NbBAK1 as Bait. We exploited the requirement for *NbBAK1* in csp22 recognition (7), which suggested a csp22-triggered complex between an unknown receptor protein and NbBAK1. For this approach, we expressed *NbBAK1b* (referred to here as *NbBAK1*) (24) from the strong 35S promoter, fused translationally to green fluorescent protein (GFP) at its C terminus (*35S:NbBAK1-GFP*). Additionally, we created a *bak1-5* variant (C508Y) (*35S:NbBAK1-5-GFP*), as AtBAK1-5 protein shows higher affinity to FLS2 than AtBAK1 (25) and hence might be a better bait in this scheme. We transformed 5-wk-old *N. benthamiana* leaves with each construct and infiltrated them with csp22 3 d later at the onset of flowering. The putative NbBAK1 protein complexes were purified from leaf extracts using immobilized anti-GFP and isolated proteins digested into peptides before analysis by liquid chromatography-mass spectrometry (LC-MS/MS) (Fig. 1*A*). Similar numbers of peptides were identified for NbBAK1 and NbBAK1-5 in both mock- and csp22-treated samples. We identified many proteins including an *N. benthamiana* homolog of BIR1 and two BIR2 homologs (*SI Appendix*, Table S1, and Fig. 1*B*) (9, 10). At the protein level, the NbBIR2 variants were 63% identical to AtBIR2. One variant was more abundant in NbBAK1 pull-downs and hence was designated NbBIR2b and the other as NbBIR2a (*SI Appendix*, Table S2). NbBIR1, NbBIR2a, and NbBIR2b were present in both mock and csp22 treatments. We further identified two LRR-RLPs that were enriched in the csp22-treated samples as CSPR candidates. We termed them receptor candidate 1 (RC1) and 2 (RC2) (Fig. 1*B* and *SI Appendix*, Table S1). We cloned the *RC1*- and *RC2*-coding regions into binary vectors under the control of the 35S promoter and fused translationally to a C-terminal 5Myc tag. We coexpressed each of these in *N. benthamiana* leaves with 35S:*NbBAK1* fused C-terminally to 3HA and 1FLAG tags (*35S:NbBAK1-3HAF*) and tested complex formation in the presence of

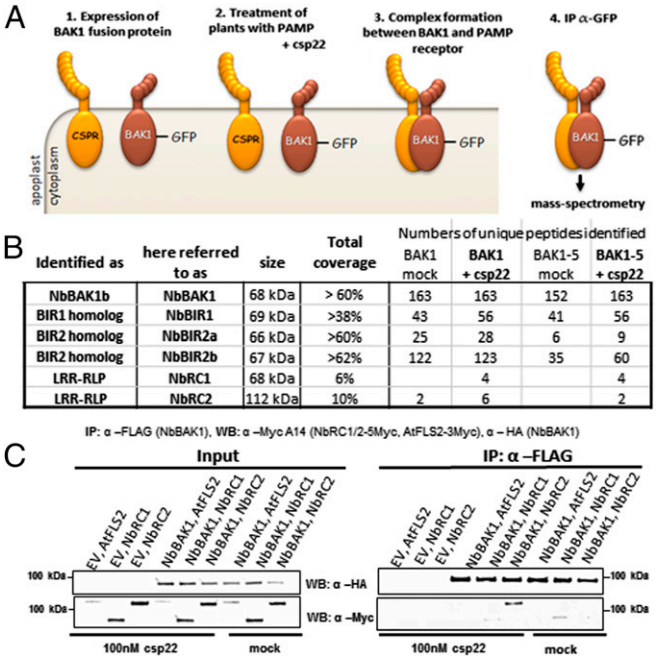

Fig. 1. Identification of NbCSPR using NbBAK1 as molecular bait. (*A*) Strategy to identify NbCSPR. *N. benthamiana* leaves were transiently transformed with *35S:NbBAK1-GFP* or *35S:NbBAK1-5-GFP* (1). Leaves were treated with csp22 (2), leading to complex formation between NbBAK1 and a hypothetical receptor protein (3). The complex was isolated using anti-GFP–conjugated beads (4), and copurifying proteins were identified by LC-MS/MS. (*B*) Selected LRR-RK and LRR-RLP proteins identified LC-MS/MS after NbBAK1 immunoprecipitation. Each protein and the number of corresponding peptides are identified (from all four experiments). "RC" stands receptor candidate for cold shock protein. (*C*) NbRC2 forms a complex with NbBAK1 in a csp22-dependent manner. *N. benthamiana* leaves were cotransformed with *35S:NbBAK1-3HAF* or EV and one of *pAtFLS2:AtFLS2-3Myc*, *35S:NbRC1-5Myc*, or *35S:NbRC2-5Myc*. Three days postinfiltration, infiltrated leaves were treated with sterile water (mock) or 100 nM csp22 for 15 min before harvesting the tissue. NbBAK1-3HAF was recovered by anti-FLAG pull-down, and immunoprecipitates were probed with anti-Myc and anti-HA western blots after gel electrophoresis. (*Left*) The input fractions. (*Right*) Immunoprecipitated fractions (IP).

csp22 by coimmunoprecipitation (coIP) experiments. Using anti-FLAG to recover NbBAK1, and probing the complexes by anti-HA and anti-Myc western blots, we found that, in contrast to the MS results, RC1 was constitutively associated with NbBAK1. On the other hand, RC2 copurified with NbBAK1 only after csp22 treatment, and not after treatment with water or flg22 (*SI Appendix*, Fig. S3*A*, and Fig. 1*C*). AtFLS2, RC1, and RC2 associated with NbBAK1-5 independently of csp22 (*SI Appendix*, Fig. S3*B*). Similar results were observed for the interaction between AtBAK1-5 and AtFLS2 (25). We concluded that RC2 is likely an RLP required for CSP-mediated PTI in *N. benthamiana* and from here on refer to it as NbCSPR, for *N. benthamiana* RLP REQUIRED FOR CSP22 RESPONSIVENESS. The predicted NbCSPR protein contains an N-terminal signal peptide, 28 extracellular tandem LRRs, and a transmembrane domain followed by a short cytoplasmic tail (*SI Appendix*, Fig. S4). CSP responsivness was identified initially in *Nicotiana tabacum* suspension cultures (22), and correspondingly we identified a homolog to *NbCSPR* in *N. tabacum* (*NtCSPR*) (*SI Appendix*, Fig. S5). We also identified *NbCSPR* sequence homologs in other *Solanaceae*, including potato (*Solanum tuberosum*), *Solanum commersonii*, *Nicotiana sylvestris*, *Nicotiana tomentosiformis*, *Petunia hybrida*, *Physalis peruviana*, and *Withania somnifera* (*SI Appendix*, Fig. S5). Tomato leaves respond to the csp15 peptide lacking the first seven amino acids of csp22 (22), but despite this, we were unable to identify a clear *NbCSPR* sequence-homolog in tomato (blast.ncbi.nlm.nih.gov/Blast.cgi and

https://solgenomics.net/tools/blast/). *A. thaliana* does not respond to csp22 and, correspondingly, we were unable to identify an NbCSPR homolog in *A. thaliana*.

NbCSPR Forms a Complex with csp22 and Is Required for csp22 Responses. To test if csp22 and NbCSPR can associate, we purified csp22-GST and flg22-GST from *Escherichia coli BL21* cells and purified NbCSPR-3HAF from *N. benthamiana* leaf extracts by anti-FLAG IP. Both csp22-GST and flg22-GST were biologically active at the concentration (500 nM) used for the association assay, as estimated by their abilities to induce ROS in *N. benthamiana* (*SI Appendix*, Fig. S6*A*). We mixed bead-bound NbCSPR with 500 nM csp22 expressed as a fusion with the GST protein (csp22-GST). After washing the beads, we found that csp22-GST was retained on the NbCSPR-bound beads (*SI Appendix*, Fig. S6*B*). NbCSPR did not associate with flg22-GST, nor with csp22-GST when 10 μM free csp22 peptide was added for competition (*SI Appendix*, Fig. S6*C*). We cannot, however, exclude the possibility that purification of NbCSPR from *N. benthamiana* coisolated additional proteins involved in the interaction with csp22. To investigate the requirement for *NbCSPR* in csp22 responses, we generated gene fragments corresponding to nucleotides 2–299 (*TRV:NbCSPRa*) and 300–1,001 (*TRV:NbCSPRb*) of the ORF and cloned them into a tobacco rattle virus (TRV) vector for virus-induced gene silencing (VIGS) (*SI Appendix*, Table S3) (26). Plants silenced for *NbCSPR* (*TRV:NbCSPRa* and *TRV:NbCSPRb*), but not those silenced for the control gene *GFP* (*TRV:GFP*), showed reduced csp22 responses, including diminished ROS production, activation of MAPKs, and up-regulation of PIG expression (Fig. 2 *A*–*C*). Silencing of *NbCSPR* did not affect flg22 responses (*SI Appendix*, Fig. S6 *D*–*G*). We detected the activation of only one MAPK in silenced plants treated with PAMPs, as reported previously (27). Successful silencing was confirmed by reduced *NbCSPR* mRNA levels (*SI Appendix*, Fig. S6*F*) and lack of detectable NbCSPR protein after transient transformation of *TRV:NbCSPRa*/*b* plants with *35S:NbCSPR-3HAF* (*SI Appendix*, Fig. S6*G*). The *TRV:NbCSPRa* construct was used for all subsequent experiments and is referred to as *TRV:NbCSPR* from here on.

NbCSPR Does Not Require NbSOBIR1 for csp22 Responses. The LRR-RK NbSOBIR1 may be generally required for RLP function through direct interaction, perhaps by providing an intracellular signaling component to the complex (15, 28). Indeed, we found that, when overexpressed in *N. benthamiana*, NbCSPR copurified with NbSOBIR1 in pull-down experiments, but AtFLS2 did not (*SI Appendix*, Fig. S7 *A* and *B*). In agreement, we found that after overexpression in *N. benthamiana*, NbSOBIR1 can form a complex with NbBAK1 after csp22 treatment (*SI Appendix*, Fig. S7*C*). Only a very weak interaction was detected after mock treatment, which may be due to *Agrobacterium*-mediated transformation. Thus, NbSOBIR1 and NbBAK1 likely associate in a csp22-induced manner. In agreement with the constitutive association of NbCSPR and NbBAK1-5, NbSOBIR1 can form a constitutive complex with NbBAK1-5 (*SI Appendix*, Fig. S7*C*). Despite this, cosilencing of *NbSOBIR1* and its close homolog *NbSOBIR1-like* (*TRV:NbSOBIR1+SOBIR-like*) (11) in *N. benthamiana* only slightly reduced the accumulation of transiently expressed NbCSPR (*SI Appendix*, Fig. S7*D*). *TRV:NbSOBIR1+SOBIR-like* plants were also not impaired in csp22- or flg22-induced production of ROS, MAPK activation, or PIG up-regulation (*SI Appendix*, Fig. S7 *G* and *H*). In fact, in *TRV:NbSOBIR1+SOBIR-like* plants, PIGs were induced to a higher extent by csp22 or flg22 treatment by comparison with *TRV:GFP* plants. Successful silencing was confirmed through reduced *NbSOBIR1* and *NbSOBIR1-like* mRNA levels and the lack of Avr4/Cf4-mediated cell death in *TRV:NbSOBIR1+SOBIR-like* plants (11) (*SI Appendix*, Fig. S7 *I* and *J*). We thus suggest the existence of an unknown protein(s) that acts redundantly to *NbSOBIR1* and *NbSOBIR1-like* in csp22-triggered immune signaling.

***NbCSPR* Confers Responsiveness to csp22 in Transgenic *A. thaliana* Plants Dependent on *AtBAK1/AtBKK1*.** Next, we tested if interfamily transfer of NbCSPR can confer csp22 recognition to a previously nonresponsive species. We first transformed *A. thaliana* Col-0 protoplasts with *35S:NbCSPR-3HA* to test for csp22-induced MAPK activation. Wild-type Col-0 protoplasts were blind to the PAMP, whereas *NbCSPR*-expressing protoplasts activated MAPKs in a csp22-dependent manner. Coexpression of NbSOBIR1 intensified the csp22-dependent MAPK activation (*SI Appendix*, Fig. S8). To further substantiate this, we generated stable transgenic *35S:NbCSPR-5Myc A. thaliana* Col-0 plants. We obtained five transgenics, but only one of these, IS-01, expressed NbCSPR-5Myc protein to a detectable level. We measured csp22-dependent responses in this line, including ROS production, seedling growth inhibition (SGI), and MAPK activation. IS-01 developed a weak ROS burst in response to csp22 that was absent in the empty vector line (IS-00). The profile of ROS production was aberrant compared with *N. benthamiana* leaf discs (Fig. 3 *A* and *B*), suggesting that NbCSPR is not properly regulated in *A. thaliana*, which might be related to the low frequency of productive transformation. In addition, we found that IS-01 plants but not control plants showed weak activation of MAPK after 5 and 15 min (Fig. 3*C*), a small but significant SGI in response to the elicitor (Fig. 3*D*) and up-regulation of *PATHOGENESIS-RELATED GENE 1* (*PR1*) expression, a late defense marker also up-regulated by flg22 and elf18 treatment (29, 30) (*SI Appendix*, Fig. S8*E*). In agreement with the *N. benthamiana* data, csp22-dependent MAPK activation in *A. thaliana* protoplasts expressing *NbCSPR* was absent in the *bak1-5 bkk1-1* double mutant, but present in the *sobir1-12* mutant (*SI Appendix*, Fig. S8). Of note, we always observed that NbCSPR accumulated to lower levels in *bak1-5 bkk1-1* protoplasts, which may also partially explain the reduced MAPK activation in response to csp22 in these protoplasts. Flg22 activated MAPKs in Col-0 and *sobir1-12* but not *bak1-5 bkk1-1* protoplasts expressing *NbCSPR* in the same experiments. Overall, the data corroborate our findings in *N. benthamiana* and support a model in which csp22 induces PTI in a manner that depends on protein complexes containing NbCSPR and BAK1 (or BKK1), potentially with SOBIR1 and/or other protein(s) with similar function.

NbCSPR Confers Age-Related Resistance to Bacterial Pathogens and Restricts *Agrobacterium*-Mediated Transformation of *N. benthamiana* in Flowering Plants. To test the relevance of *NbCSPR* for antibacterial immunity, we silenced *NbCSPR* or *NbFLS2* in *N. benthamiana* using VIGS and infected 4- or 6-wk-old silenced

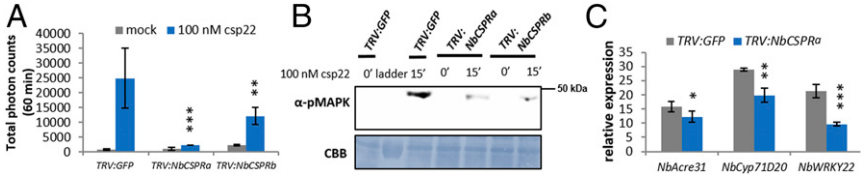

Fig. 2. NbCSPR is required for csp22-dependent responses. *NbCSPR* is required for csp22-dependent responses as determined by VIGS of *N. benthamiana* plants and measuring (*A*) ROS production, (*B*) activation of MAPKs, and (*C*) up-regulation of PIG expression. Graphed data are ±SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (pairwise Student's *t* test comparing *TRV:NbCSPR* to *TRV:GFP* plants; $n = 8$ for ROS; $n = 6$ for qRT-PCR). Experiments were performed at least three times and representative results are shown.

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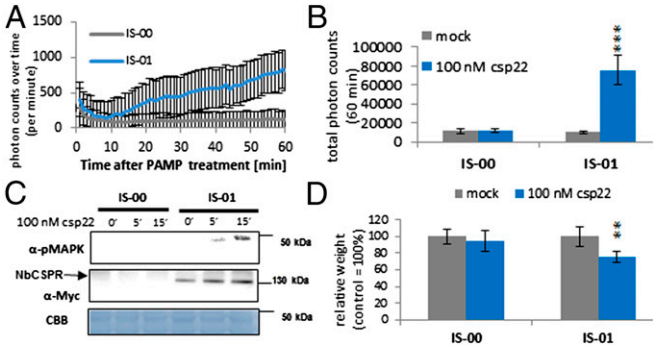

Fig. 3. *NbCSPR* confers recognition of csp22 in *A. thaliana*. Overexpression of *NbCSPR* in stable transgenic *A. thaliana* Col-0 plants (IS-01) leads to csp22-dependent responses, including (*A* and *B*) production of ROS, (*C*) MAPK activation, and (*D*) SGI. Graphed data are ±SEM, $^{**}P < 0.01$, $^{***}P < 0.001$ (pairwise Student's *t* test comparing IS-01 to EV plants (IS-00, $n = 8$). Experiments were performed at least twice and representative results are shown.

plants with adapted and nonadapted *Pseudomonas syringae* strains. Both *FLS2- and NbCSPR-*silenced plants supported more than 1 log growth of the adapted pathogen *P. syringae* pv. *tabaci* (*Pta*) 6605 (Fig. 4*A*) compared with control plants silenced for *GFP*. This is consistent with *NbCSPR* playing an important role in antibacterial immunity. To test this further, we inoculated silenced plants with a mutant strain deficient in the type-III secretion system (*Pta* 6605 *hrcC*$^{-}$) (Fig. 4*B*). Again, bacteria grew significantly more on *N. benthamiana* plants silenced for *NbFLS2* or *NbCSPR* than on plants silenced for *NbGFP*. Finally, to test the relative contribution of NbCSPR to bacterial immunity in the absence of flagellin recognition, we inoculated silenced plants with the *Pta 6605 fliC*$^{-}$ mutant lacking the flagellin gene (31). Accordingly, bacterial growth was not increased on *NbFLS2*-silenced plants but showed a small but significant increase in 6-wk-old plants silenced for *NbCSPR* (Fig. 4*C*). This effect was not seen on 4-wk-old plants (*SI Appendix*, Fig. S9*A*). To test a role for NbCSPR against nonadapted pathogens, we inoculated silenced plants with *P. syringae* pv. *phaseolicola* 1448A (32) (Fig. 4*D*). The weak growth of this strain was significantly higher on plants silenced for *NbFLS2* or *NbCSPR* compared with plants silenced for *GFP*. We also found that *NbCSPR* contributed to bacterial resistance when transferred into *A. thaliana*. The stable transgenic lines IS-00 and IS-01 were spray-infected with adapted *P. syringae* pv. *tomato* DC3000 bacteria. Plants expressing *NbCSPR* (IS-01) showed slightly reduced bacterial growth relative to the empty vector (EV) (IS-00) line (Fig. 4*E*). Taken together, our data show that NbCSPR is an important component of antibacterial immunity. Flowering *N. benthamiana* plants are recalcitrant to *Agrobacterium*-mediated transformation (33). As *A. tumefaciens* contains CSP genes that are likely elicitor-active (*SI Appendix*, Fig. S9*B*), we tested if *NbCSPR* restricts *Agrobacterium*-mediated transformation. Four-week-old plants silenced for *GFP*, *NbFLS2*, or *NbCSPR* were equally transformable by *A. tumefaciens* as judged by expression of an *intron-GUS* marker gene (*SI Appendix*, Fig. S9*C*). Older plants were minimally transformable after silencing for *GFP* or *NbFLS2* (Fig. 4*F*). Strikingly, *NbCSPR*-silenced plants showed much higher GUS activity comparable to expression in young plants. Similarly, transient expression of an arbitrary gene (N2) encoding the amino acids 1–242 of the *Solanum lycopersicum* Prf protein (34) (*35S:N2-3HAF*) in flowering plants revealed greater N2 accumulation in plants silenced for *NbCSPR* relative to those silenced for *GFP* (*SI Appendix*, Fig. S9*D*). N2 protein levels were unchanged by gene silencing in younger plants (*SI Appendix*, Fig. S9*E*). Greater resistance of older plants to *Agrobacterium*-mediated infiltration may be related to *NbCSPR* up-regulation of about twofold in 6-wk-old relative to 4-wk-old plants, an effect that was not seen for *NbFLS2* (*SI Appendix*, Fig. S9*F*). Our data demonstrate a role for *NbCSPR* in restricting genetic transformation by *A. tumefaciens*.

Potentiation of csp22 Responses by flg22 Pretreatment. In contrast to wild-type *Pta* 6605, NbCSPR restricted growth of *Pta* 6605 *fliC*$^{-}$ only in 6-wk-old plants. We thus investigated the role of flagellin perception on csp22-mediated immune responses. We found that prior flg22 treatment caused higher csp22-dependent production of ROS, PIG up-regulation, and MAPK activation including activation of a second MAPK (Fig. 5 *A–C*). Interestingly, both csp22-induced ROS and MAPK assays showed decreases after csp22 pretreatment, which may be a similar phenomenon to the refractory period of diminished FLS2-mediated responses after initial flg22 perception (35). Treatment of *N. benthamiana* leaves with 100 nM csp22 or the unrelated PAMP chitin at 100 μg/mL significantly up-regulated *NbCSPR* expression, but this effect was far higher upon treatment with 100 nM flg22. Conversely, flg22 treatment up-regulated *NbFLS2* to only a small extent, whereas its induction by csp22 was negligible (*SI Appendix*, Fig. S10 *A* and *B*). PTI responses induced by flg22 were not increased by prior csp22 treatment (*SI Appendix*, Fig. S10 *C–E*). Similarly, prior flg22 treatment caused higher elf18-dependent production of ROS in *A. thaliana*, but elf18 pretreatment did not result in increased flg22-mediated ROS production (*SI Appendix*, Fig. S10 *E* and *F*). Overall, prior flg22 treatment increased csp22 responses in *N. benthamiana* and elf18 responses in *A. thaliana* but not vice versa, perhaps consistent with the fact that flagellin is an external PAMP, and CSP and EF-Tu are internal.

Discussion

We report here identification of a LRR-RLP required for csp22 responses using a previously undescribed biochemical approach. *NbCSPR* encodes a previously undescribed LRR-RLP that can form a constitutive complex with SOBIR1. NbCSPR associates with NbBAK1

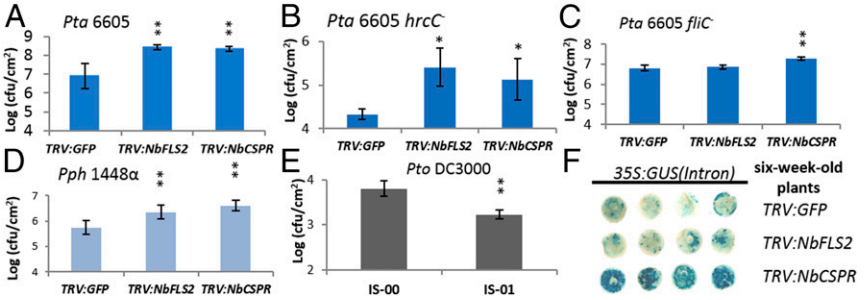

Fig. 4. *NbCSPR* contributes to antibacterial immunity. *N. benthamiana* plants were silenced for *GFP*, *NbFLS2*, or *NbCSPR* before infection by dipping into *P. syringae* suspensions. Silenced plants were infected with (*A*) *P. syringae* pv. *tabaci* (*Pta*) 6605, (*B*) *Pta* 6605 *hrcC*$^{-}$, (*C*) *Pta* 6605 *fliC*$^{-}$, and (*D*) *P. syringae* pv. *phaseolicola* 1448A (*Pph*). Graphed data are ±SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ (pairwise Student's *t* test comparing *TRV:NbFLS2* or *TRV:NbCSPR* to *TRV:GFP* plants; $n = 6$). (*E*) Stable transgenic Col-0 plants transformed with *35S:EV-5Myc* (IS-00) or *35S:NbCSPR-5Myc* (IS-01) were spray-infected with *P. syringae* pv. *tomato* DC3000 bacteria. Plants were dip- and spray-infected using a bacterial suspension of 5×10^7 cfu/mL, and samples were taken after 3 d. (*F*) Transformation of 6-wk-old *N. benthamiana* plants is restricted by *NbCSPR*. *N. benthamiana* plants were silenced for *GFP*, *NbFLS2*, or *NbCSPR* before infiltration with *A. tumefaciens* GV3101 pMp90 carrying a *35S:intron-GUS* construct (21). Leaves were harvested 2 d postinfiltration, and GUS activity was detected by GUS staining. Blue color indicates transformation with the *GUS* gene. All experiments were performed at least twice, and representative results are shown.

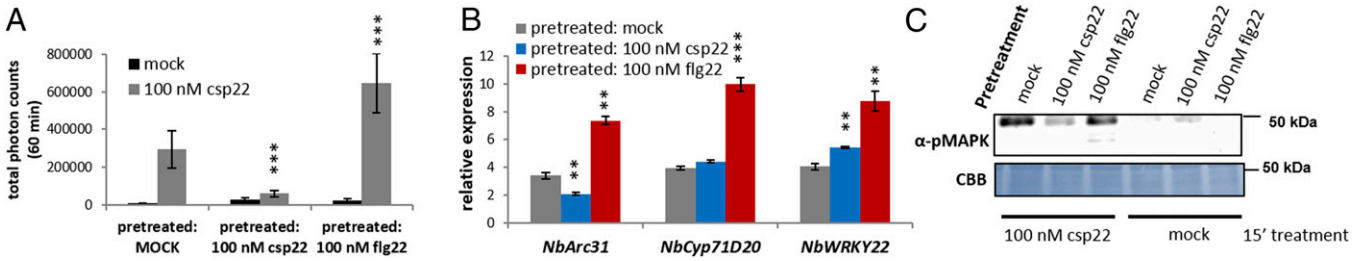

Fig. 5. flg22 perception potentiates csp22 responsiveness in 4-wk old *N. benthamiana* plants. Increase in csp22-dependent (*A*) ROS production, (*B*) expression of PIG relative to mock-treated controls, and (*C*) MAPK activation in *N. benthamiana* leaves after flg22 pretreatment. flg22 was removed and replaced with sterile water before treatment with csp22. Graphed data are ±SEM, $^{**}P < 0.01$, $^{***}P < 0.001$ (pairwise Student's *t* test comparing flg22 or csp22 pretreated plants to mock-pretreated plants; $n = 8$ for ROS; $n = 6$ for qRT-PCR). Experiments were performed at least twice, and representative results are shown.

after elicitation and is required for immunity to bacterial pathogens. It is active in 6-wk-old plants where it restricts the growth of adapted and nonadapted pathogens and transient transformation by *A. tumefaciens*. Interestingly, our results suggest a mechanism in which PAMP perception is coordinated temporally as prior flagellin perception potentiates NbCSPR-mediated immunity in 4-wk-old plants.

We used a proteomics approach to identify LRR-RKs or LRR-RLPs that depend on common complex components such as BAK1. In contrast to previous pull-down experiments, our method aims to identify LRR-RKs and LRR-RLPs that form a complex with BAK1 in response to a specific ligand. It is well established that BAK1 is a central regulator of immunity through interaction with LRR-RKs or RLPs after PAMP perception (6, 7). We showed previously that csp22-dependent ROS production is *NbBAK1*-dependent and as such predicted a csp22-induced interaction between NbBAK1 and an unknown LRR-RK or RLP. Through purifying NbBAK1-GFP (or NbBAK1-5-GFP) after csp22 treatment, we identified known interactors of BAK1 including *N. benthamiana* homologs of AtBIR1 and AtBIR2 (9, 10). Notably, we did not detect a release of either NbBIR2 variant from NbBAK1 after csp22 treatment as has been reported for AtBIR2 (10). This may reflect a biological difference or was perhaps due to NbBAK1 overexpression. Most importantly, we identified two proteins that were enriched in csp22-treated samples. Subsequent coIP analysis confirmed our LC-MS/MS results and showed the csp22-dependent association of one of these proteins with NbBAK1. Overall, the approach was successful and offers a general strategy to identify BAK1-associated proteins that play specific roles in PAMP perception/responsiveness. Genetic tests showed that *NbCSPR* is required for csp22-dependent responses and antibacterial immunity. Plants silenced for *NbCSPR* were deficient in csp22-triggered ROS production, MAPK activation, and up-regulation of PIGs. Consistent with this, the silenced plants were more susceptible to infection by adapted and nonadapted *P. syringae* pathogens. Silencing of *NbCSPR* allowed a similar increase in bacterial growth as silencing NbFLS2. Moreover, plants silenced for *NbCSPR* were transformed more efficiently by *A. tumefaciens* than *TRV:GFP* plants, but this effect was not seen for *NbFLS2*. This result reflects the fact that *A. tumefaciens* possesses a conserved CSP protein containing the csp22 motif (*SI Appendix*, Fig. S9*B*), but its variant flagellin is not recognized (19). Recognition of *A. tumefaciens* CSP may suggest why NbCSPR peptides were recovered from NbBAK-GFP preparations before csp22 treatment. Restriction of *Agrobacterium*-mediated transformation by NbCSPR is not unexpected because EFR also limits transformation in *A. thaliana* and transgenic *N. benthamiana* (21).

The nonresponsive species *A. thaliana* initiated csp22-dependent production of ROS, MAPK activation, and SGI after transformation with *35S:NbCSPR-5Myc*. The transfer of *NbCSPR* to protoplasts of *A. thaliana* allowed csp22-dependent MAPK activation in the transformed cells, whereas wild-type Col-0 protoplasts were blind to the PAMP. Importantly, NbCSPR-mediated signaling in *A. thaliana* protoplasts required AtBAK1 and/or its close paralogue AtBKK1. Finally, we showed that NbCSPR expressed in *N. benthamiana* tissue associated with csp22-GST and that this interaction was abrogated when excess free csp22 peptide was used in competition for binding. We therefore conclude that NbCSPR is required for csp22 responses in *N. benthamiana* and may be the csp22 receptor.

NbSOBIR1 is required for accumulation and functionality of multiple RLPs, perhaps by stabilizing the respective receptor or by providing transmembrane signaling capability (11–15, 28). Although NbSOBIR1 associated with NbCSPR and also with NbBAK1 after csp22 treatment, silencing of *NbSOBIR1* and its close homolog *NbSOBIR1-like* only weakly affected accumulation of transiently expressed NbCSPR. This may explain why neither *NbSOBIR1* nor its close homolog *NbSOBIR1-like* were required for csp22-induced responses. We used the *TRV:NbSOBIR1+NbSOBIR-like* silencing construct that targets both genes (11). Cosilencing of *NbSOBIR1* and *NbSOBIR1-like* was confirmed by qRT-PCR and the lack of *Avr4*/*Cf4*-induced hypersensitive response, as shown previously (11). The same plants exhibited all csp22-induced responses. We further found that SOBIR1 was dispensable for NbCSPR-dependent csp22 responsiveness in the *A. thaliana sobir1-12* protoplasts. This may be due to the strong NbCSPR protein levels detected during these experiments. Overall, our data suggest that SOBIR1 is involved in NbCSPR function, but that additional proteins may act redundantly to NbSOBIR1 in csp22-mediated immunity.

CSP responses were far greater in plants that were transitioning to flowering than in younger plants. This may be due to an increase in *NbCSPR* expression or several other untested regulatory mechanisms. The difference is biologically significant because older plants were more resistant to *Pta* bacteria lacking flagellin and were recalcitrant to transformation by *A. tumefaciens*. Both effects were reversed by *NbCSPR* silencing. Despite the fact that csp22 generally exhibited weaker PTI responses than flg22 (7, 19, 22), plants silenced for *NbCSPR* showed strikingly similar levels of bacterial growth compared with *NbFLS2*-silenced plants. This was true for adapted and nonadapted *P. syringae*. However, we cannot exclude differential silencing levels of each gene. The *Pta fliC*$^{-}$ strain that cannot activate FLS2 showed similar growth on *NbFLS2*-silenced plants to *TRV:GFP* plants, as expected. Growth of this strain was slightly but significantly higher in *NbCSPR*-silenced plants, again demonstrating a role for NbCSPR in antibacterial immunity. Likewise, the efficiency of *Agrobacterium*-mediated transformation in plants silenced for *GFP* or *NbFLS2* was similar, whereas *NbCSPR*-silenced plants showed both strongly enhanced GUS activity and accumulation of the N2 protein after transient transformation. Similarly, resistance to *Xanthomonas oryzae* pv. *oryzae* mediated by the rice LRR-RKs Xa21 and Xa3/Xa26 is developmentally regulated (36, 37). Our data further show that younger plants can compensate for their deficiency in csp22 perception by up-regulating *NbCSPR* expression in response to flg22. This potentiated all csp22-induced responses tested here and may explain why *NbCSPR* does not restrict the growth of the flagellin-deficient strain *Pta* 6605 *fliC*$^{-}$ in 4-wk-old plants. This is an important observation because one potential interpretation is that flagellin and CSP perception occur sequentially. This would accord with the fact that flagellin is an

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external PAMP that is immediately visible to the infected plant, whereas CSP and EF-Tu are internal and must be released before host perception. This model implies that FLS2 identifies the invading microbe as bacterial. Consistent with this view, both eukaryotic pathogens and *N. benthamiana* itself express proteins with conserved CSDs, and a protein with a CSD from *Nicotiana sylvestris* elicited a defense response on *N. tabacum* cells (22). Hence, an additional level of regulation may be necessary for appropriate deployment of CSP recognition, perhaps also to avoid an auto-immune response. We further speculate that the importance of developmental regulation of NbCSPR might be related to the difficulty in recovering *35S:NbCSPR A. thaliana* transgenics.

In summary, we have used a proteomics procedure to purify and identify a previously undescribed LRR-RLP, CSPR, involved in antibacterial immunity. NbCSPR transfer to *A. thaliana* confers responsiveness to the bacterial PAMP csp22, which suggests that NbCSPR directly recognizes csp22. We cannot exclude completely that NbCSPR acts as a coreceptor for the csp22-binding determinant. Our data suggest that transfer of *NbCSPR* to plant species otherwise blind to CSP may be useful for conferring resistance to bacterial diseases in agriculture. In addition, knocking out or silencing the expression of the *NbCSPR* will improve transient *Agrobacterium*-mediated transformation of *N. benthamiana* for industrial and experimental uses.

Materials and Methods

De novo identification of NbBAK1-associated proteins by LC-MS/MS following anti-GFP immunoprecipitation: NbBAK1-GFP was overexpressed in *N. benthamiana*. Proteins were extracted and coIP performed as described using anti-GFP (ChromoTek) (38). LC-MS/MS, software processing, and peptide identification were performed as described (39) with the difference that a combined Sol genomics/TGAC *N. benthamiana* predicted protein database was used for protein identification.

All other materials and methods can be found in *SI Appendix, SI Materials and Methods*.

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