Extracellular pyridine nucleotides trigger plant systemic immunity through a lectin receptor kinase/BAK1 complex

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Systemic acquired resistance (SAR) is a long-lasting broad-spectrum plant immunity induced by mobile signals produced in the local leaves where the initial infection occurs. Although multiple structurally unrelated signals have been proposed, the mechanisms responsible for perception of these signals in the systemic leaves are unknown. Here, we show that exogenously applied nicotinamide adenine dinucleotide (NAD\(^+\)) moves systemically and induces systemic immunity. We demonstrate that the lectin receptor kinase (LecRK), LecRK-VI.2, is a potential receptor for extracellular NAD\(^+\) (eNAD\(^+\)) and NAD\(^+\) phosphate (eNADP\(^+\)) and plays a central role in biological induction of SAR. LecRK-VI.2 constitutively associates with BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 (BAK1) in vivo. Furthermore, BAK1 and its homolog BAK1-LIKE1 are required for eNAD(P)\(^+\) signaling and SAR, and the kinase activities of LecR-VI.2 and BAK1 are indispensable to their function in SAR. Our results indicate that eNAD\(^+\) is a putative mobile signal, which triggers SAR through its receptor complex LecRK-VI.2/BAK1 in Arabidopsis thaliana.
All eukaryotes possess a sophisticated immune system to defend against microbial pathogens. Whereas animals employ both innate and adaptive immunities, plants lack adaptive immunity and strictly rely on innate immunity. Every plant cell is capable of using the so-called two-tiered innate immune system to detect potential microbial pathogens and mount immune responses. In the local leaves where the initial infection occurs, plant cells utilize pattern recognition receptors (PRRs) to sense conserved features on/in the invading microbes, which are named pathogen-associated molecular patterns (PAMPs), and activate PAMP-triggered immunity (PTI). Successful pathogens employ effectors to dampen PAMP signaling. Resistant plants in turn exploit resistance proteins to detect the presence of pathogen effectors, inducing effector-triggered immunity. These plant–pathogen battles often cause injuries to the plant cells, resulting in release of host-derived damage-associated molecular patterns (DAMPs) that further potentiate immunity through PRRs.

Pathogen perturbation in the local leaves induces production of mobile signals that are transported to the uninfect ed parts (the systemic leaves) of the plant, leading to the activation of a long-lasting form of immunity known as systemic acquired resistance (SAR) that confers resistance to subsequent infections by a broad spectrum of pathogens. A group of structurally unrelated molecules have been proposed as SAR mobile signals, which include the lipid transfer protein Defective in Induced Resistance1 (DIR1), salicylic acid (SA) and its derivative methyl SA, dehydroabietinal, azelaic acid (AZA), glycerol-3-phosphate (G3P), piperocid acid (Pip) and its derivative N-hydroxy-Pip (NHP), and monoterpenes. In addition, nitric oxide (NO) and reactive oxygen species (ROS) have been implicated in the SAR signaling amplification loop.

As shown in Fig. 1a, b, the amounts of NAD(P) in the water surrounding the Psm-infiltrated leaf disks were significantly higher than those surrounding the mock (10 mM MgCl2)-infiltrated leaf disks in the first 36 h. To test whether NAD(P) could leak out of intact leaves, detached leaves were submerged in water with the petioles above the water surface. As shown in Supplementary Fig. 1a, b, NAD(P) levels in leaf disks continuously dropped significantly at 36 and 48 hour. The slower kinetics of NAD(P) leakage from intact leaves than from leaf disks may be attributed to the process of diffusing out of the apoplast. Nevertheless, these results indicate that significant amounts of NAD(P) leaked into the extracellular space after Psm infection.

To mimic the eNAD(P)+ dynamics during pathogen infection, we infiltrated three lower leaves on each Arabidopsis plant with 0.4 mM NAD+ or 0.8 mM NAD+ every 12 hours for a total of four times. Approximately 5 hours after the last infiltration, the systemic leaves were collected for analysis of the induction of Pathogenesis-Related gene1 (PR1), a marker gene of SAR. To test NAD(P)+-induced systemic resistance, the systemic leaves were challenged with Psm and the in planta bacterial growth was determined 3 days later. Meanwhile, Psm-triggered biological induction of SAR was used as the positive control. To this end, three lower leaves on each plant were infiltrated with Psm. Two days later, the systemic leaves were either collected for PR1 analysis or challenge-inoculated with Psm for resistance test. As shown in Fig. 1c, d, treatment of lower leaves with NAD(P)+ significantly induced expression of PR1 and resistance to Psm in the systemic leaves, though the induction levels were significantly lower than those triggered by Psm, which is consistent with the notion that multiple signal molecules are involved in SAR activation. Taken together, these results suggest that eNAD(P)+ may function as an SAR signal molecule in Arabidopsis.

NAD(P)+ may act in the infiltrated (local) leaves or be transported to the systemic leaves to trigger resistance. To test whether eNAD(P)+-triggered systemic resistance is associated with translocation of eNAD(P)+ from local to systemic leaves, an NAD+ solution (1 mM unlabeled NAD+ plus 6.25 nm 32P-NAD+) was infiltrated into lower leaves on Arabidopsis and N. benthamiana plants (Fig. 1e, f). Twenty-four hours later, the systemic leaves were collected for autoradiographic detection of 32P. As shown in Fig. 1e, f, infiltration of 32P-NAD+ resulted in movement of the radioactivity into the systemic leaves in both Arabidopsis and N. benthamiana. To define the portion of radioactivity that moves systemically, Arabidopsis leaves were infiltrated with 6.25 nm 32P-NAD+ (without unlabeled NAD+) and the radioactivity in the local and systemic leaves was quantified. As shown in Supplementary Fig. 2a, ~10.8% and 6.1% of the radioactivity moved into the systemic leaves in the wild type and the 35S:CD38 transgenic plants, respectively. Furthermore, whether NAD+ per se moves systemically was tested by paper chromatography. As shown in Supplementary Fig. 2b,
although three bands were detected in the extracts of local and systemic leaves, the predominant band has the same size as 32P-NAD+. This result supports the hypothesis that eNAD+ can be transported from local to systemic leaves. Interestingly, the signal intensity of the potential 32P-NAD+ band in the extract of 35 S:CD38 systemic leaves was significantly reduced (Supplementary Fig. 2b), indicating that the movement of 32P-NAD+ in the 35 S:CD38 plants was inhibited. This observation is line with the previously reported result that SAR induction is partially compromised in 35 S:CD38 plants.

LecRK-VI.2 is a potential receptor for eNAD(P)+. To convincingly demonstrate that eNAD(P)+ is an SAR signal molecule, it is crucial to identify its receptors and test their role in SAR responses. We have previously identified LecRK-L8 as a potential eNAD+-binding receptor in Arabidopsis. LecRK-I.8 binds NAD+ with a dissociation constant (Kd) of 436.5 ± 104.8 nM. Surprisingly, NADP+ did not compete for binding of LecRK-I.8 with 32P-NAD+. In line with this result, mutations in LecRK-I.8 did not affect NADP+-induced immune responses. Furthermore, the lecrk-I.8 mutations compromised basal immunity but had no effect on biological induction of SAR. These results prompted us to identify eNADP+-binding receptors. We have previously isolated transferred DNA (T-DNA) insertion lines for a group of receptor-like genes that were induced by exogenous NAD+22. We suspected that NAD+ might also induce eNADP+-binding receptor genes and thus tested exogenous NADP+-induced Psm resistance in the T-DNA insertion lines. As shown in Supplementary Fig. 3a, NADP+-induced resistance was clearly reduced in the T-DNA insertion line SALK_070801, which harbors a T-DNA insertion in the gene At5g01540 that encodes LecRK-VI.2. We then tested the transcript levels of LecRK-VI.2 in SALK_070801 and two more T-DNA insertion lines, SAIL_1146_B02 and SAIL_796_E11. Basal transcript levels of LecRK-VI.2 in the T-DNA insertion lines were ~33–48% of those in the wild type, indicating that all three T-DNA insertion lines are knockdown mutants of LecRK-VI.2 (Supplementary Fig. 3b, c). SALK_070801 has previously been named lecrk-VI.2-1, and we thus named SAIL_1146_B02 and SAIL_796_E11 lecrk-VI.2-2 and lecrk-VI.2-3, respectively (Supplementary Fig. 3b). Although previous work has shown that NAD+-induced resistance to Psm was not affected in the three lecrk-VI.2 alleles, NAD+-induced expression of PR1, PR2, and PR5 were all significantly inhibited with the exception of PR5 in lecrk-VI.2-3 (Fig. 2a–c). Importantly,
Fig. 2 Exogenous NAD(P)+-induced local defense responses in the lecrk-VI.2 mutants and binding of NAD(P)+ to LecRK-VI.2. a–c Exogenous NAD(P)+-induced local expression of PR1, PR2, and PR5 in the wild-type (WT), lecrk-VI.2, and dom1-3 plants. Leaves of 4-week-old soil-grown plants were infiltrated with 0.2 mM NAD+, 0.4 mM NADP+ or H2O. The infiltrated leaves were collected 20 hr later. Total RNA was extracted and subjected to qPCR analysis. Expression levels were normalized against the constitutively expressed UBQ5. Data represent the mean ± SD of three biological replicates. Asterisks denote significant differences between the inductive in the mutants and that in the wild type (*p < 0.05; **p < 0.01; ***p < 0.001; two-way ANOVA with Sidak’s test). Upper line: induction by NADP+; lower line: induction by NAD+. d Exogenous NADP+-induced local resistance in the wild-type, lecrk-VI.2, and dom1-3 plants. Leaves of 4-week-old soil-grown plants were infiltrated with 0.4 mM NADP+ or H2O. Five hr later, the infiltrated leaves were inoculated with a Psm suspension (OD600 = 0.001). Three d later, eight leaves were collected to examine the growth of the pathogen. Data represent the mean ± SD of eight biological replicates. Asterisks denote significant differences between the induction in the mutants and that in the wild type (***p < 0.001; two-way ANOVA with Sidak’s test). e Binding of 32p-NADP+ to recombinant MBP, MBP-eLecRK-VI.2, and MBP-eDORN1 proteins. Approximately 5 μg recombinant proteins were used for each binding assay. Data represent the mean ± SD of three experiments. Different letters denote significant differences (p < 0.05; one-way ANOVA with Tukey’s test). f Saturation binding assay for LecRK-VI.2. Recombinant MBP-eLecRK-VI.2 proteins were incubated with the indicated concentrations of 32p-NADP+ for 30 min. Free NADP+ was removed by washing. Data were plotted as a specific binding. The dissociation constant (Kd) was calculated by one site specific binding saturation model using GraphPad Prism 7 (www.graphpad.com). The experiment was repeated four times with similar results and results from a representative experiment were presented. g Competitive binding assay for LecRK-VI.2. Samples containing 250 nM of 32p-NADP+ in the presence of 100 nM to 1 mM of unlabeled nucleotides were assayed for specific binding of 32p-NADP+. Data were plotted as a specific binding with SD of three experiments. The 50% inhibition concentration (IC50) values were calculated in GraphPad Prism 7 using the one site Fit loglC50 competition model. h Binding of 32p-NADP+ to the microsomal fractions of the wild-type (WT) and lecrk-VI.2-2 mutant plants. Samples containing 250 nM 32p-NADP+ in the absence (total) or presence of 1000-fold unlabeled (cold) NAD2+ or NADP+ were assayed for binding of 32p-NAD2+. Data represent the mean ± SD of three experiments. Different letters denote significant differences (p < 0.05; one-way ANOVA with Tukey’s test).
NADP\(^+\)-induced PR gene expression and Psm resistance were significantly suppressed in all three alleles but not in the extracellular ATP receptor mutant *dorn1-3*\(^{26}\) (Fig. 2a–d). These results suggest that LecRK-VI.2 may play a more important role in eNADP\(^+\) signaling than in eNAD\(^+\) signaling in the local leaves.

We then tested whether LecRK-VI.2 binds NAD\(^+\) using 32P-NAD.\(^+\). The extracellular domain (amino acids (AAs) 23-310) of LecRK-VI.2 (eLecRK-VI.2) was expressed and purified from *Escherichia coli* as a recombinant maltose-binding protein (MBP) fusion (Supplementary Fig. 3d) and subjected to binding assays with 32P-NAD\(^+\) (AA 22–288) proteins. MBP-eLecRK-VI.2 exhibited a typical saturation curve for NAD\(^+\) binding with a KD of 786.7 ± 127.6 nM (Fig. 2f).

To test whether LecRK-VI.2 binds NAD\(^+\), we compared the abilities of unlabeled NAD\(^+\), NAD\(^P\), and ATP to compete for binding of 32P-NAD\(^+\). Binding of 32P-NAD\(^+\) to MBP-eLecRK-VI.2 was effectively competed by unlabeled NAD\(^+\) (50% inhibition concentration, IC\(_{50}\) = 1,887 nM) and NADP\(^+\) (IC\(_{50}\) = 945 nM), but was not competed by ATP (IC\(_{50}\) > 100,000 nM) (Fig. 2g). As the IC\(_{50}\) of NADP\(^+\) is approximately half of that of NAD\(^+\), the relative binding affinity of NAD\(^+\) is slightly higher than that of NAD\(^P\), which is in agreement with the compromised NADP\(^+\) responsiveness in the *lecrk-VI.2* mutants (Fig. 2a–d). Furthermore, the total NAD\(^+\) binding activity in the membrane fractions of the *lecrk-VI.2* mutant was significantly lower than that in the wild type, and the binding of 32P-NAD\(^+\) to the membrane fractions was also effectively competed by both unlabeled NAD\(^+\) and NADP\(^+\) (Fig. 2h).

Note that the reduced binding of 32P-NAD\(^+\) to the membrane fractions of *lecrk-VI.2* was further competed by unlabeled NAD\(^+\) and NADP\(^+\), indicating the existence of other NAD(P)\(^+\)-binding proteins. Taken together, these results indicate that LecRK-VI.2 is a potential receptor for both NAD\(^+\) and NADP\(^+\). In agreement with this conclusion, the LecRK-VI.2 gene is induced by both NAD\(^+\) and NADP\(^+\) (Supplementary Fig. 3e).

**LecRK-VI.2 is required for NAD(P)\(^+\)-induced systemic immunity.** To test whether LecRK-VI.2 is essential for exogenous NAD(P)\(^+\) to trigger systemic resistance, we infiltrated three lower leaves on each *Arabidopsis* plant with 0.4 mM NAD\(^+\) or 0.8 mM NADP\(^+\) every 12 hours for a total of four times. About 5 hours after the last infiltration, the systemic leaves were either collected for analysis of the induction of *PR1* or challenge-inoculated with *Psm* to test NAD(P)\(^+\)-induced systemic resistance. As shown in Fig. 3a, b, although mutations in LecRK-VI.2 did not significantly inhibit NAD(P)\(^+\)-induced *PR1* gene expression, NAD(P)\(^+\)-induced resistance to *Psm* in the systemic leaves was completely blocked in the mutants plants. This result indicates that LecRK-VI.2 is required for exogenous NAD(P)\(^+\)-induced systemic resistance and further supports LecRK-VI.2 being an eNAD(P)\(^+\) receptor.

To test the necessity of the well-documented SAR pathway genes in eNAD(P)\(^+\) signaling, we first monitored exogenous NAD(P)\(^+\)-induced resistance to *Psm* in the local leaves of various SAR mutants. Interestingly, except *fad2* (*fatty acid desaturase 2*)\(^{27}\), only mutations affecting SA accumulation, including *sid2* (SA induction deficient)\(^{28}\), *eds5* (enhanced disease susceptibility 5)\(^{29}\), *eds3* (enhanced resistance 3)\(^{30}\), *pad4* (phytoalexin deficient 4)\(^{31}\), and *npr1* (non race-specific disease resistance 1)\(^{32}\), significantly inhibited NAD(P)\(^+\)-induced *Psm* resistance (Supplementary Fig. 4a–d). The *npr1-3* (nonexpressor of PR genes 1-3) mutation, which largely blocks SA signaling\(^{33}\), significantly suppressed NAD\(^+\)- but not NADP\(^+\)-induced resistance (Supplementary Fig. 4a), which is consistent with the result obtained with the *npr1-1* allele\(^{19}\). These results indicate that eNAD(P)\(^+\) activates SA-dependent signaling and may function downstream or independently of *ALD1* (AGD2-Like Defense response protein 1)\(^{34}\), *FMO1* (Flavin-dependent Monoxygenase)\(^{35}\), *AZI1* (Azelaic acid Induced)\(^{36}\), *NHO1* (NONHOST1)\(^{37}\), *PEPR1* (PEPp Receptor1)\(^{27}\), and *SD1* (Suppressor of Fatty acid desaturase Deficiency)\(^{38}\) in the local leaves.

We further tested exogenous NAD(P)\(^+\)-induced systemic resistance to *Psm* in the systemic leaves of *sid2, aed1*, *fmo1*, and *nho1*, which do not accumulate SA, the putative SAR mobile signals Pip, NHP, and G3P, respectively, and *npr1, azi1*, and *pep1 pepr2*, which do not respond to SA, the putative SAR signal molecule AzA, and Pep peptide elicitors, respectively. To this end, three lower leaves on each plant were infiltrated with 0.4 mM NAD\(^+\) or 0.8 mM NADP\(^+\) every 12 hours for a total of four times. Five hours after the last infiltration, the systemic leaves were challenge-inoculated with *Psm* to test NAD(P)\(^+\)-induced systemic resistance. As shown in Fig. 3c, NAD(P)\(^+\)-induced systemic resistance against *Psm* was significantly suppressed in *sid2* and *npr1*, but not in *aed1, fmo1, nho1, azi1, and pep1 pepr2*, indicating that eNAD(P)\(^+\)–triggered systemic signaling requires SA and NPR1, but not Pip, NHP, G3P, AzA, and Pep peptide elicitors.

We have previously shown that high concentrations (> 1 mM) of NAD(P)\(^+\) significantly induce SA accumulation\(^{19}\). To test whether the concentrations used above could induce SA accumulation, free SA levels in leaf tissues treated with 0.4 mM NAD\(^+\) or 0.8 mM NADP\(^+\) were measured. As shown in Supplementary Fig. 5a, 0.4 mM NAD\(^+\) and 0.8 mM NADP\(^+\) did not significantly induce free SA accumulation in the treated leaves, suggesting that basal SA is both necessary and sufficient for eNAD(P)\(^+\)-induced defense signaling. On the other hand, previous microarray analysis indicated that SA treatment quickly (1 hour) induces LecRK-VI.2 expression\(^{40}\). We confirmed this result by quantitative PCR (qPCR) and further revealed that the induction depends on the transcription activator NPR1 (Supplementary Fig. 5b, c). These results indicate that SA signaling positively regulates LecRK-VI.2 expression, suggesting a signaling amplification loop comprising SA, NPR1, and LecRK-VI.2.

**LecRK-VI.2 is required for biological induction of SAR.** If eNAD(P)\(^+\) is an SAR signal molecule and LecRK-VI.2 is a receptor of eNAD(P)\(^+\), mutations in LecRK-VI.2 should block biological induction of SAR. To test this hypothesis, we infiltrated three lower leaves on each plant with either 10 mM MgCl\(_2\) (−SAR) or *Psm* (+SAR). Two days later, expression of *PR1, PR2*, and *PR5* in the systemic leaves was analyzed. As shown in Fig. 4a–c, induction of the three commonly used SAR marker genes was significantly suppressed in the *lecrk-VI.2* mutants, but not in *dorn1-3*. To test SAR activation-induced resistance in the *lecrk-VI.2* plants, we challenge-inoculated the systemic leaves with *Psm* and determined the bacterial growth after 3 days. As shown in Fig. 4d, SAR activation induced strong resistance in the wild-type and *dorn1-3* plants, whereas the resistance was significantly inhibited in the *lecrk-VI.2* mutants. We further conducted a microarray experiment to compare SAR activation-induced transcriptome changes in *lecrk-VI.2* and wild type (National Center for Biotechnology Information Gene Expression Omnibus series number GSE121886). Systemic leaf tissues were collected two days after inoculation of the lower leaves with *Psm*, as significant systemic increases in PR gene expression and resistance were observed at this time point\(^{41}\) (Fig. 4a–d). Genes that were induced or suppressed twofold or higher with a low q value (≤0.05) by SAR activation in *lecrk-VI.2* and the wild type
were identified and compared. As shown in Fig. 4e, f, a total of 564 and 151 genes in the wild type were up- and downregulated, respectively, whereas only 55 and 26 genes in lecrk-VI.2-2 were up- and downregulated, respectively. In other words, transcript levels of >90 and 82% of the genes that were induced and suppressed, respectively, in the wild type were not significantly changed in the lecrk-VI.2-2 mutant. These results indicate that the eNAD(P)⁺-binding receptor LeCRK-VI.2 is a crucial component in the SAR signaling pathway.

BAK1 and BKK1 are required for eNAD(P)⁺ signaling. It has previously been shown that LeCRK-VI.2 has a positive role in PTI via constitutive association with the leucine-rich repeat receptor kinase (LRR-RK) PRR FLAGELLIN-SENSING2 (FLS2)\(^25,42\). Upon flagellin perception, FLS2 recruits its co-receptors BAK1 and BAK1-LIKE1 (BKK1), which phosphorylate their interacting receptor-like cytoplasmic kinase Botrytis-Induced Kinase1 (BIK1) to initiate the PTI signaling\(^33-48\). To test whether these well-established PTI signaling components function in eNAD(P)⁺ signaling, we tested exogenous NAD(P)⁺-induced immune responses in the bak1-5\(^{49}\), bkk1-1\(^{50}\), bak1-5 bkk1-1\(^{49}\), fls2 efr (elongation factor Tu receptor)\(^31,52\), and bkk1-1\(^{45}\) mutants. As shown in Fig. 5a, b and Supplementary Fig. 6a, b, NAD(P)⁺-induced PR gene expression and Psm resistance were not significantly altered in the fts2 efr and bkk1 mutants, whereas the induction was differentially affected by the bak1-5 and bkk1 mutations. NAD⁺-induced expression of PRI and PR2 was significantly inhibited in bak1-5, bkk1, and bak1-5 bkk1, but NAD⁺-induced expression of PR5 and resistance to Psm was only significantly repressed in the bak1-5 bkk1 double mutant. On the other hand, NAD⁺-induced PR gene expression and Psm resistance were significantly suppressed in both single and double mutants. These results indicate that BAK1 and BKK1 play overlapping and independent roles in the eNAD(P)⁺ signaling pathways.

BAK1 and BKK1 are required for biological induction of SAR. Since BAK1 and BKK1 function in eNAD(P)⁺ signaling, they may be required for SAR. To test this hypothesis, three lower leaves on each plant were infiltrated with either 10 mM MgCl₂ (−SAR) or Psm (+SAR). Two days later, expression of PRI,
PR2, and PR5 in the systemic leaves was analyzed. Induction of the three PR genes was significantly reduced in the bak1-5, bkk1, and bak1-5 bkk1 mutants (Fig. 5c and Supplementary Fig. 6c, d). We also challenge-inoculated the systemic leaves with Psm and determined in planta bacterial titers after 3 days. As shown in Fig. 5d, SAR activation induced strong resistance in the wild-type, fls2 cfr, and bkl1 plants, whereas the resistance was significantly compromised in the bak1-5, bkk1, and bak1-5 bkk1 mutants. We have included the bak1-5 mutant in the microarray experiment described above (Fig. 4e, f) to compare SAR activation-induced transcriptome changes in bak1-5 with those in lecrk-VI.2-2 and the wild type. Genes that were induced or suppressed twofold or higher with a low q value (≤ 0.05) by SAR activation in bak1-5 were identified and compared with those in the wild type and lecrk-VI.2-2. As shown in Fig. 5e, f, a total of 221 and 121 genes were up- and
downregulated, respectively, in bak1–5, which fall between the numbers of genes that were up- and downregulated in the wild type and lecrk-VI.2–2. Furthermore, the majority of the genes that were induced or suppressed twofold or higher with a low q value (≤0.05) in the wild type were induced or suppressed to a smaller extent in bak1–5 and the smallest extent in lecrk-VI.2–2 (Supplementary Fig. 7 and Supplementary Data 1). These results indicate that the bak1–5 mutation suppressed SAR activation-induced signaling, but the strength of the suppression was not as strong as that in lecrk-VI.2–2, which may be attributed to the presence of BKK1 that might have some redundant functions with BAK1 in biological induction of SAR (Fig. 5d).

**LecRK-VI.2 and BAK1 form a complex in vivo.** As both LecRK-VI.2 and BAK1 function in eNAD(P)+ signaling and biological induction of SAR, they may form a protein complex. To test this hypothesis, we examined the interaction between the two proteins using three different approaches. First, we used pull-down assays to investigate possible interaction between the cytoplasmic kinase domains (KDs) of LecRK-VI.2 and BAK1 (LecRK-VI.2KD and BAK1KD, respectively). As shown in Fig. 6a and Supplementary Fig. 8a, amylose beads pulled down both MBP-LecRK-VI.2KD and GST-BAK1KD from a mixture of purified MBP-LecRK-VI.2KD and GST-BAK1KD, but only pulled down MBP from a mixture of purified MBP and GST-BAK1KD or MBP-LecRK-VI.2KD from a mixture of purified MBP-LecRK-VI.2KD and GST. This result indicates that LecRK-VI.2KD can physically interact with BAK1KD in vitro. Second, we employed the co-immunoprecipitation (Co-IP) technique in N. benthamiana to test possible association of LecRK-VI.2 with BAK1 in vivo. As shown in Fig. 6b, anti-FLAG beads precipitated both LecRK-VI.2-1.0 and BAK1-1.0.
Fig. 5 Exogenous NAD(P)^+–induced local immune responses and biological induction of SAR in several PTI mutants. a Exogenous NAD(P)^+–induced local PR1 expression in the wild-type (WT), bak1-5, bkk1, bak1-5 bkk1, fts2 erf, and bikl plants. Leaves of 4-week-old soil-grown plants were infiltrated with 0.2 mM NAD\(^+\), 0.4 mM NADP\(^+\) or H\(_2\)O. The infiltrated leaves were collected 20 hr later. Total RNA was extracted and subjected to qPCR analysis. Expression levels of PR1 were normalized against the constitutively expressed UBQ5. Data represent the mean ± SD of three biological replicates. Asterisks denote significant differences between the induction in the mutants and that in the wild type (*p < 0.05, **p < 0.01, ***p < 0.001; two-way ANOVA with Sidak’s test). Upper line: induction by NADP\(^+\); lower line: induction by NAD\(^+\). b Exogenous NAD(P)^+–induced local resistance in the wild-type, bak1-5, bkk1, bak1-5 bkk1, fts2 erf, and bikl plants. Leaves of 4-week-old soil-grown plants were infiltrated with 0.2 mM NAD\(^+\), 0.4 mM NADP\(^+\) or H\(_2\)O. Five hr later, the infiltrated leaves were inoculated with a Psm suspension (OD\(_{600}\) = 0.001). Three d later, eight leaves were collected to examine the growth of the pathogen. Data represent the mean ± SD of eight biological replicates. Asterisks denote significant differences between the induction in the mutants and that in the wild type (*p < 0.05, **p < 0.01; two-way ANOVA with Sidak’s test). c Expression of PR1 in the systemic leaves of the wild-type, bak1-5, bkk1, bak1-5 bkk1, fts2 erf, and bikl plants with or without SAR induction. Three lower leaves on each 4-week-old soil-grown plant were infiltrated with 10 mM MgCl\(_2\) or a Psm suspension (OD\(_{600}\) = 0.002). Two d later, systemic leaves were collected for PR1 expression analysis by qPCR. Data represent the mean ± SD of three biological replicates. Asterisks denote significant differences between the induction in the mutants and that in the wild type (*p < 0.05; two-way ANOVA with Sidak’s test). d Biological induction of SAR in the wild-type, bak1-5, bkk1, bak1-5 bkk1, fts2 erf, and bikl plants. Three lower leaves on each 4-week-old soil-grown plant were infiltrated with 10 mM MgCl\(_2\) or a Psm suspension (OD\(_{600}\) = 0.002). Two d later, two systemic leaves were challenge-inoculated with Psm (OD\(_{600}\) = 0.001). Three d later, eight leaves were collected to examine the growth of the pathogen. Data represent the mean ± SD of eight biological replicates. Asterisks denote significant differences between the induction in the mutants and that in the wild type (**p < 0.01; ***p < 0.001; two-way ANOVA with Sidak’s test). e Overlaps among the genes that were up- or downregulated in the systemic leaves of the wild-type, bak1-5, and lecrk-VL2-2 plants. Plants were treated as in c. Total RNA extracted from the systemic tissues was subjected to microarray analysis. Genes with an absolute fold change ≥ 2 and a q value ≤ 0.05 were compared with obtain overlapped genes among the wild-type, bak1-5, and lecrk-VL2-2 plants.

Fig. 6 Physical association between LecRK-VL2 and BAK1. a In vitro maltose-binding protein (MBP) pull-down assay of LecRK-VL2KD interaction with BAK1KD. Recombinant MBP or MBP-LecRK-VL2KD was incubated with GST-BAK1KD and pulled down with amylase resin beads. Input and bead-bound proteins were analyzed by immunoblotting with monoclonal anti-MBP and anti-GST antibodies. b Co-immunoprecipitation (Co-IP) analysis of LecRK-VL2-FLAG association with BAK1-FLAG in N. benthamiana. Total proteins (input) of N. benthamiana leaves transiently co-expressing LecRK-VL2-FLAG and BAK1-FLAG, or transiently expressing BAK1-FLAG alone, were immunoprecipitated with anti-FLAG affinity agarose beads and the precipitates were analyzed by immunoblotting with monoclonal anti-FLAG and anti-GST antibodies. The leaves were treated with (+) or without (−) 0.8 mM NADP\(^+\) for 10 min. c Co-IP analysis of LecRK-VL2-HA association with BAK1-FLAG in Arabidopsis. Total proteins of Arabidopsis leaves from the BAK1:BAK1-FLAG/LecRK-VL2-FLAG transgenic plants were immunoprecipitated with monoclonal anti-FLAG antibody/protein G plus agarose and the precipitates were analyzed by immunoblotting with polyclonal anti-FLAG and monoclonal anti-HA antibodies. The leaves were treated with (+) or without (−) 0.8 mM NADP\(^+\) for 10 min.

FLAG and BAK-GFP (green fluorescence protein) from N. benthamiana transiently co-expressing the LecRK-VL2-FLAG and BAK1-FLAG fusion proteins, and NADP\(^+\) treatment did not affect the amount of precipitated BAK1-FLAG. In contrast, anti-FLAG beads did not precipitate BAK1-FLAG from N. benthamiana transiently expressing BAK1-FLAG only (Fig. 6b), indicating that BAK1-FLAG did not bind nonspecifically to the anti-FLAG beads. Similarly, anti-FLAG beads precipitated LecRK-VL2-FLAG and...
BAK1-GFP but not plasma membrane localized LT16B-GFP\textsuperscript{53} (Supplementary Fig. 8b), excluding the possibility of nonspecific interaction with GFP. These results indicate that LecRK-VI.2-HA and BAK1-GFP constitutively associate with each other when transiently expressed in \textit{N. benthamiana}. Finally, we created stable transgenic \textit{Arabidopsis} plants co-expressing LecRK-VI.2-HA and BAK1-GFP by transforming a LecRK-VI.2/LecRK-VI.2-HA construct into the previously characterized BAK1:BAK1-GFP plants\textsuperscript{54}. The LecRK-VI.2/LecRK-VI.2-HA construct was also transformed into wild type to generate plants expressing LecRK-VI.2-HA only as a control. Co-IP experiments were conducted using these plants to test the association between LecRK-VI.2-HA and BAK1-GFP. As shown in Fig. 6c, anti-GFP/protein G plus agarose precipitated both BAK1-GFP and LecRK-VI.2-HA from the BAK1:BAK1-GFP/LecRK-VI.2/LecRK-VI.2-HA plants, and NADP\textsuperscript{+} treatment did not increase the amount of LecRK-VI.2-HA. On the other hand, anti-GFP/protein G plus agarose did not precipitate any LecRK-VI.2-HA from plants carrying the LecRK-VI.2/LecRK-VI.2-HA transgene only. Taken together, these results demonstrate that LecRK-VI.2 and BAK1 constitutively form a protein complex in vivo.

To confirm that LecRK-VI.2 and BAK1 function through a protein complex in eNAD(P)\textsuperscript{+} signaling and SAR, we generated a lecrk-VI.2 bak1 double mutant by crossing lecrk-VI.2-2 with bak1-5 and tested its responses to exogenous NADP\textsuperscript{+} treatment and biological induction of SAR. As shown in Supplementary Fig. 9a, b, NADP\textsuperscript{+}-induced and SAR activation-conferring resistance to \textit{Psm} was comparable in the lecrk-VI.2 bak1 double mutant and the lecrk-VI.2-2 single mutant. This result indicates no additive effect between the lecrk-VI.2-2 and bak1-5 mutations, supporting that LecRK-VI.2 and BAK1 function in SAR by constituting a protein complex.

The importance of the kinase activity of LecR-VI.2 and BAK1.

To determine whether phosphorylation is involved in LecRK-VI.2/BAK1-mediated signaling, we tested if the kinase activities of LecRK-VI.2 and BAK1 are required for their functions in eNADP\textsuperscript{+} signaling and biological induction of SAR. To this end, a point mutation was introduced into the LecRK-VI.2 LecRK-VI.2-HA construct to replace a conserved Asp (D) residue at the position 494 of the catalytic loop HRD motif with an Asn (N) residue. The resulting construct (LecRK-VI.2 lecrk-VI.2 (D494N)-HA) and the wild-type construct were then transformed into the lecrk-VI.2-2 background. As shown in Fig. 7a–c and Supplementary Fig. 10a, the wild-type construct LecRK-VI.2 LecRK-VI.2-HA almost completely complemented the defects of lecrk-VI.2-2 in NADP\textsuperscript{+}-induced \textit{PR} gene expression and \textit{Psm} resistance as well as biological induction of SAR, whereas the LecRK-VI.2 lecrk-VI.2 (D494N)-HA construct did not rescue any of these defects. For BAK1, we transformed the 35S:BAK1-GFP construct and a 35S: baki(K317E)-GFP construct into the bak1-5 background. The K317E mutation has been shown to abolish the kinase activity of BAK1\textsuperscript{54,55}. Although it has been shown that BAK1-GFP is not fully functional in PTI responses and bak1-5 is a dominant-negative mutation\textsuperscript{49,56}, the 35S:BAK1-GFP construct largely complemented the bak1-5 mutant phenotypes including reduced induction of \textit{PR} gene expression and \textit{Psm} resistance by NADP\textsuperscript{+} as well as compromised SAR (Fig. 7d–f and Supplementary Fig. 10b). To exclude the possibility of seed contamination or transgene modification, we confirmed the bak1-5 genetic background of the transgenic plants and the inability of BAK1-GFP to complement bak1-5 in flg22-induced ROS burst (Supplementary Fig. 11a, b). Therefore, C-terminal-tagged BAK1 is still functional in NADP\textsuperscript{+} signaling and SAR. On the other hand, the 35S:saki(K317E)-GFP construct did not complement any of the bak1-5 phenotypes (Fig. 7d–f and Supplementary Fig. 10b). Taken together, these results demonstrate that the kinase activities of both LecRK-VI.2 and BAK1 are required for eNADP\textsuperscript{+}-induced immune responses and biological induction of SAR, suggesting that phosphorylation is involved in LecRK-VI.2/BAK1-mediated eNADP\textsuperscript{+} and SAR signaling.

LecRK-VI.2 is required for flg22-induced systemic immunity.

It has been shown that LecRK-VI.2 contributes to PTI responses including flg22 (a 22-amino acid peptide corresponding to the N terminus of bacterial flagellin)-induced MPK3/6 activation, callose deposition, and stomatal closure\textsuperscript{25}. Consistently, the lecrk-VI.2-1 mutant plants are highly sensitive to \textit{P. syringae pv. tomato} DC3000 when plants are dip inoculated\textsuperscript{25}. To explore possible...
Fig. 7  The necessity of the kinase activities of LecRK-VI.2 and BAK1 in eNAD(P)+ signaling and SAR. **Exogenous NAD(P)+-induced local expression of **PR1, **PR2, and **PR5 in the wild-type (WT), lecrk-VI.2-2, LeckrK-VI.2-HA, and LeckrK-VI.2-lecrk-VI.2(D494N)-HA plants. Leaves of 4-week-old seed-grown plants were infiltrated with 0.4 mM NADP+ or H2O. The infiltrated leaves were collected 20 hr later. Total RNA was extracted and subjected to qPCR analysis. Expression levels were normalized against the constitutively expressed UBQ5. Data represent the mean ± SD of three biological replicates. Asterisks denote significant differences between the induction in the mutant or transgenic plants and that in the wild type (**p < 0.01, *** p < 0.001; two-way ANOVA with Sidak’s test). b Exogenous NADP+-induced local resistance in the wild-type, lecrk-VI.2-2, LeckrK-VI.2-HA, and LeckrK-VI.2-lecrk-VI.2(D494N)-HA plants. Leaves of 4-week-old soil-grown plants were infiltrated with 0.4 mM NADP+ or H2O. Five hr later, the infiltrated leaves were inoculated with a Psm suspension (OD600 = 0.001). Three d later, eight leaves were collected to examine the growth of the pathogen. Data represent the mean ± SD of eight biological replicates. Asterisks denote significant differences between the induction in the mutant or transgenic plants and that in the wild type (**p < 0.001; two-way ANOVA with Sidak’s test). c Biological induction of SAR in the wild-type, lecrk-VI.2-2, LeckrK-VI.2-LeckrK-VI.2-HA, and LeckrK-VI.2-lecrk-VI.2(D494N)-HA plants. Three lower leaves on each 4-week-old soil-grown plant were infiltrated with 10 mM MgCl2 or a Psm suspension (OD600 = 0.002). Two d later, two systemic leaves were challenged-inoculated with Psm (OD600 = 0.001). Three d later, eight leaves were collected to examine the growth of the pathogen. Data represent the mean ± SD of eight biological replicates. Asterisks denote significant differences between the induction in the mutant or transgenic plants and that in the wild type (**p < 0.001; two-way ANOVA with Sidak’s test). d Exogenous NADP+-induced local expression of **PR1, **PR2, and **PR5 in the wild-type, bak1-5, 35S::BAK1-GFP, and 35S::SbAK1(K317E)-GFP plants. The experiment was conducted as in a. Data represent the mean ± SD of three biological replicates. Asterisks denote significant differences between the induction in the mutant or transgenic plants and that in the wild type (**p < 0.001; two-way ANOVA with Sidak’s test). e Exogenous NADP+-induced local resistance in the wild-type, bak1-5, 35S::SBAK1-GFP, and 35S::SbAK1(K317E)-GFP plants. The experiment was conducted as in b. Data represent the mean ± SD of eight biological replicates. Asterisks denote significant differences between the induction in the mutant or transgenic plants and that in the wild type (**p < 0.01; two-way ANOVA with Sidak’s test). f Biological induction of SAR in the wild-type, bak1-5, 35S::SBAK1-GFP, and 35S::SbAK1(K317E)-GFP plants. The experiment was conducted as in c. Data represent the mean ± SD of eight biological replicates. Asterisks denote significant differences between the induction in the mutant or transgenic plants and that in the wild type (**p < 0.01; two-way ANOVA with Sidak’s test). g22-induced local apoplastic resistance in the wild-type, Psm inflamed leaves into the extracellular space to test its resistance. As shown in Supplementary Fig. 12e, g22-induced systemic resistance was significantly inhibited in the three lecrk-VI.2 mutants. Therefore, LeckrK-VI.2 is crucial for g22-induced systemic immunity but not for g22-induced local apoplastic resistance.

Discussion

Although it has been well documented in animals that eNAD(P)+ functions in numerous physiopathological processes18, eNAD(P)+ and its signaling role in plants have not been established. The identification of the first eNAD(P)+-binding receptor in Arabidopsis has stimulated us to begin investigating this interesting molecule in depth24. Here, we identified LeckrK-VI.2 as a potential primary eNAD(P)+-binding receptor, which allowed us to uncover the LeckrK-VI.2/BAK1 complex that has a central role in the establishment of SAR in Arabidopsis.

Several lines of evidence generated in this study suggest that eNAD(P)+ may be an SAR signal molecule in Arabidopsis. First, NADP+ continuously leaks into the extracellular space during pathogen infection (Fig. 1a, b). Second, local application of physiological concentrations of NAD(P)+ induces resistance in the systemic leaves (Fig. 1c, d). Third, mutations in the potential eNAD(P)+-binding receptor LeckrK-VI.2 significantly inhibit SAR induction (Fig. 4). And fourth, BAK1 and BKK1 are required for both eNAD(P)+ signaling and SAR (Fig. 5). The above hypothesis is also supported by our previous finding that removal of eNAD(P)+ by transgenic expression of the human NAD(P)+-hydrolizing ectoenzyme CD38 partially compromises biological induction of SAR21.

Interestingly, locally applied 32P-NAD+ and/or its metabolite (s) moved systemically, although the radioactivity signal detected in the systemic leaves is much weaker than that in the local leaves (Fig. 1e, f). Approximately 10.8% of the applied radioactivity moved into the systemic leaves in wild-type Arabidopsis plants (Supplementary Fig. 2a), suggesting that only a small fraction of eNAD(P)+ accumulated in the local leaves could be transported to the systemic leaves. Similarly, only a small fraction (~7–10%) of several other putative SAR mobile signals including AzA and G3P is transported from the local to systemic leaves37. Thus, eNAD(P)+ could potentially function as an SAR mobile signal in plants. However, as exogenously added molecules often move systemically38,39, further in-depth investigations are required to conclusively determine whether and how endogenous eNAD(P)+ moves.

Treatment of various SAR mutants revealed that eNAD(P)+ functions either downstream or independently of the putative mobile signals Pip, NHP, G3P, and AzA in both local and systemic leaves (Fig. 3c and Supplementary Fig. 4a). It has been proposed that Pip, NO, ROS, AzA, and G3P function in a signaling amplification loop and that ROS mediates a systemic signal network in SAR7,15,16. Biochemically, ROS has been shown to oxidize cell membrane, leading to pore formation and membrane disintegration20,61. Thus, the ROS produced in the Pip-NO-ROS-AzA-G3P amplification loop may cause reversible or irreversible injuries to the plasma membrane, resulting in leakage of cellular NAD(P)+ into the extracellular space to activate its receptor LeckrK-VI.2. On the other hand, eNAD(P)+-induced defense signaling requires SA (Fig. 3c and Supplementary Fig. 4a), and, conversely, SA induces LeckrK-VI.2 gene expression through the coactivator NPR1 (Supplementary Fig. 5c). These results suggest a signaling amplification loop involving SA, NPR1, and LeckrK-VI.2. Nevertheless, as NAD(P)+-induced expression of PRI, an SA pathway marker gene, is relationship between eNAD(P)+ and flag22, we tested flag22-induced local apoplastic and systemic resistance in the eNAD(P)+- receptor mutants. To this end, we first generated a lecrk-1.8-lecrk-VI.2 (lecrk-1.8/VI.2) double mutant by crossing lecrk-1.8-2 with lecrk-VI.2-2 and tested its responses to exogenous NAD(P)+ treatment and biological induction of SAR. The double mutant behaved like lecrk-1.8-2 upon NAD+ treatment and like lecrk-VI.2-2 upon NADP+ treatment or SAR induction (Supplementary Fig. 12a-c), suggesting that mutations in LeckrK-1.8 and LeckrK-VI.2 inhibit eNAD+ and eNADP+ signaling, respectively. We then tested flag22-induced local apoplastic resistance in the double mutant. As shown in Supplementary Fig. 12d, 100 nm flag22 induced similar levels of resistance to Psm in the wild-type, lecrk-1.8-2, lecrk-VI.2-2, and lecrk-1.8/VI.2 plants. Finally, we examined flag22-induced systemic immunity in the lecrk-VI.2 single mutants, as the lecrk-1.8-2 mutation did not affect SAR22 (Supplementary Fig. 12c). To this end, three lower leaves on each plant were infiltrated with 200 nm flag22 every 12 hours for a total of 4 times. Five hours after the last infiltration, the systemic leaves were challenge-inoculated with Psm to test flag22-induced resistance. As shown in Supplementary Fig. 12e, flag22-induced systemic resistance was significantly inhibited in the three lecrk-VI.2 mutants. Therefore, LeckrK-VI.2 is crucial for flag22-induced systemic immunity but not for flag22-induced local apoplastic resistance.
not always correlated with NAD(P)⁺-induced disease resistance (Fig. 3a, b and 5a, b), the relationship between eNAD(P)⁺ and SA as well as other SAR signal molecules is complicated and warrants further investigation.

LecRK-VI.2 binds both NAD⁺ and NADP⁺ (Fig. 2e–h), indicating that LecRK-VI.2 is a potential receptor for both NAD⁺ and NADP⁺. Interestingly, mutations in LecRK-VI.2 did not affect NAD⁺-induced resistance to Psm in the local leaves22, but completely blocked both NAD⁻⁻ and NADP⁻⁻-induced resistance in the systemic leaves (Fig. 3b), suggesting that LecRK-VI.2 may be involved in eNAD(P)⁺-triggered SAR signal production in the local leaves and/or perceiving eNAD(P)⁺ and/or other signal molecules in the systemic leaves. Furthermore, while exogenous NAD(P)⁺-induced systemic PRI transcription was only slightly reduced in lecrk-VI.2 mutants (Fig. 3a), Psm-induced systemic PRI gene expression was almost completely abolished (Fig. 4a–c), suggesting that LecRK-VI.2 may have eNAD(P)⁺-independent functions. This is not without precedent for PRRs. For instance, the human cell-surface receptors TLR2 (Toll-like receptor 2) and TLR4 recognize a group of chemically different ligands including PAMPs and DAMPs62. Further investigation on potential ligands of LecRK-VI.2 would help reveal the activation mechanisms of this important plant immune regulator.

Mutations in LecRK-VI.2 had no effect on basal immunity against bacterial pathogens when the bacteria were inoculated by leaf infiltration25, but significantly suppressed biological induction of SAR (Fig. 4). The partial SAR defect in lecrk-VI.2 mutants may be attributed to possible redundancy in eNAD(P)⁺ perception, as the Arabidopsis genome encodes 75 LecRKs (32 G-type, 42 L-type, and 1 C-type)63. Indeed, LecRK-VI.2 is not the only receptor for eNAD(P)⁺ in Arabidopsis22. Nevertheless, ~ 90% of the transcriptome changes in the systemic leaves were suppressed in the lecrk-VI.2-2 mutant (Fig. 4e, f), indicating that LecRK-VI.2 is a key SAR signaling component responsible for the vast majority of SAR signaling triggered by Psm. In line with this conclusion, overexpression of LecRK-VI.2 has been shown to confer constitutive resistance against bacterial pathogens, which is a phenotype similar to SAR25. As LecRK-VI.2 is a plasma membrane-located transmembrane protein with an extracellular NAD(P)⁺-binding domain and a cytoplasmic kinase domain (Fig. 2e–h)64,65, these results suggest that eNAD(P)⁺-triggered LecRK-VI.2-mediated outside-in signaling is an integral constituent of the SAR signaling pathway. In support of this hypothesis, the LecRK-VI.2-associating BAK1 and its homolog BKK1, two plasma membrane-located LRR-RKs30,54,55, are also required for SAR (Fig. 5c, d).

BAK1 and BKK1 have been established as crucial co-receptors or adaptors for a number of LRR domain PRRs.66 The PRR-BAK1 immune complex formation is fine-tuned by other regulators. For instance, the malectin-like RLKs FERONIA (FER) and IMPAIRED OOMYCETE SUSCEPTIBILITY1 constitutively associate with both FLS2 and BAK1 to act as scaffolds for ligand-induced FLS2-BAK1 complex formation67,68. FER is a receptor for several Arabidopsis RAPID ALKALINIZATION FACTORS (RALFs)68,69. The constitutive association between BAK1 and FER can be strongly enhanced upon treatment with flg22, whereas binding of RALF23 to FER inhibits flg22-induced complex formation between FLS2 and BAK168. LecRK-VI.2 also constitutively associates with FLS2 and BAK170 (Fig. 6), but is dispensable for flg22-induced FLS2-BAK1 complex formation, suggesting a distinct mechanism underlying the function of LecRK-VI.2 in PTI. As a potential eNAD(P)⁺ receptor, binding of eNAD(P)⁺ may enhance its function in the FLS2-BAK1 immune complex. Indeed, exogenous NAD⁺ induces expression of the PTI marker genes WRKY53 and FRK1 as well as production of ROS30,22.

The interplay between eNAD(P)⁺ and flg22 (and other PAMPs) signaling is complex and deserves further attention. Recognition of flg22 by FLS2 results in PTI responses including ROS burst, which could potentially damage the plasma membrane, contributing to accumulation of eNAD(P)⁺. eNAD(P)⁺ in turn could potentiate flg22 signaling through the LecRK-VI.2-FLS2 complex. Indeed, flg22-induced MPK3/6 activation, callose deposition, and stomatal closure are inhibited in lecrk-VI.2-mutant plants25, though the contribution of eNAD(P)⁺ in this process remains to be determined. On the other hand, the potential roles of eNAD(P)⁺ in PAMP signaling and SAR are clearly distinct. First, only BAK1 and BKK1, but not FLS2, are required for SAR (Fig. 5c, d), indicating that the LecRK-VI.2-BAK1 complex, but not the LecRK-VI.2-FLS2 complex, has a major role in SAR. In agreement with this idea, flg22-induced systemic immunity is compromised in the lecrk-VI.2 mutants, whereas flg22-induced local apoplastic resistance is not significantly affected by the mutations (Supplementary Fig. 12d, e). Furthermore, the BAK1-GFP fusion is not functional in PTI responses26, but is active in NAD(P)⁺ signaling and SAR (Fig. 7d–f), indicating that the LecRK-VI.2-BAK1 complex-mediated eNAD(P)⁺ signaling is likely independent or downstream of flg22 signaling.

The kinase activities of both LecRK-VI.2 and BAK1 are essential to their function in eNAD(P)⁺ signaling and SAR (Fig. 7), suggesting that binding of eNAD(P)⁺ to LecRK-VI.2 may trigger transphosphorylation between LecRK-VI.2 and BAK1, which in turn may phosphorylate downstream target(s). A potential target is the serine/threonine kinase SNF1-RELATED PROTEIN KINASE2.8 (SnRK2.8), which has been shown to function in SAR via phosphorylating NPR171. NPR1 is a master transcription coactivator in plants and nuclear localization is required for its function in plant immunity33. In the systemic leaves, SAR activation-induced redox changes switch NPR1 from an oligomer to monomers and SnRK2.8 phosphorylates monomeric NPR1 at Ser-589 and Thr-373 to facilitate its nuclear import, which is a critical step in SAR induction71.

Induction of SAR comprises four stages: signal generation, signal translocation, signal perception, and SAR execution. Among the four stages, signal perception in the systemic leaves is the least understood3,24. In this study, we uncovered a putative SAR mobile signal eNAD(P)⁺ and its potential receptor LecRK-VI.2 and demonstrate that the LecRK-VI.2/BAK1 complex has a pivotal role in SAR. Identification of the precise relationship between eNAD(P)⁺ and other SAR signal molecules as well as the downstream targets of the LecRK-VI.2/BAK1 complex will shed further light on the SAR signaling process.

**Methods**

**Arabidopsis growth.** Arabidopsis thaliana seeds were sown on autoclaved soil (Sunshine MVP, Sun Gro Horticulture, Agawam, MA, USA) and cold-treated at 4 °C for 3 days. After germination, seedlings were grown at 22–24 °C under a 16-h-light/8-h-dark regime for 2 weeks. Seedlings were then transferred individually into pots containing the autoclaved soil. Four-week-old plants were used for NAD(P)⁺ treatment and Psm inoculation.

**Pseudomonas culture.** Pseudomonas syringae pv. maculicola ES4326 (Psm) was grown at 28 °C in King’s B medium containing 50 μg mL⁻¹ streptomycin under shaking at 220 rpm. Bacteria in overnight log-phase cultures were precipitated by centrifugation at 500 × g for 5 min, re-suspended in 10 mM MgCl₂, and diluted to different final OD600 levels for leaf infiltration experiments.

**NAD(P) leakage.** Three fully expanded leaves on each 4-week-old soil-grown Arabidopsis plant were infiltrated from the abaxial side with 10 mM MgCl₂ (mock) or a Psm suspension (OD600 = 0.002). Ten min after the treatment, 30 leaf disks (~7 mm in diameter) for each treatment were removed and washed twice for a total of 50 min. Sets of 10 leaf disks each were then placed in 5 mL fresh water in a test tube. NAD(P) concentrations in the water were measured over time following an...
enzymatic cycling assay protocol. One hundred μL of the water surrounding the leaf disks was added into a 1.5 mL microcentrifuge tube containing 0.1 mL Bicine-NaCl buffer (10 mM, pH 8.0), followed by addition of each treatment: (dissodium salt, 40 μM), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, 4.2 μM), phenazine ethosulphate (PES, 16.6 μM), and ethanol (5.0 μL) for the determination of NAD or 0.05 μL of glucose-6-phosphate (G6P, 50 μM) for the determination of NADP. After adjusting the total volume to 1 mL by adding H2O, the microcentrifuge tube was kept at 37°C for 5 min. The reaction was started by adding 0.02 mL of alcohol dehydrogenase (500 units mL⁻¹) for NAD or G6P dehydrogenase (35 units mL⁻¹, for NADP). After incubated at 37°C for 10 min, the reaction mixture was transferred to a microcentrifu and the absorbance at 570 nm was measured with a Beckman DU640 spectrophotometer (Beckman).

Plant treatment with NAD⁺ and NADP⁺. β-Nicotinamide adenine dinucleotide sodium salt (NAD⁺-Na; Sigma-Aldrich) and β-NAD⁺ phosphate sodium (NADP⁺-Na; Sigma-Aldrich) were dissolved in water, and the pH of the resulting solutions was adjusted to 6.0 using 0.1 M NaOH. The solution (0.2 mM NAD⁺ or 0.4 mM NADP⁺) was infiltrated from the abaxial side into Arabidopsis leaves of 4-week-old soil-grown plants using a 1-μL needleless syringe. Distilled water was used as the negative control. The infiltration was conducted between 10 and 11 AM. For NAD(P)⁺-induced defense gene expression, the infiltrated leaf tissues were collected 20 hr after the treatment for RNA analysis. For NAD(P)⁺-induced Psm resistance, the infiltrated leaves were inoculated with Psm 5 hr after the NAD(P)⁺ treatment as described below (“assessment of plant resistance to Psm”). For SAR induction, three lower leaves on each Arabidopsis plant were infiltrated with H2O (mock), 0.4 mM NAD⁺, or 0.8 mM NADP⁺ every 12 hr for a total of four times. The first infiltration was performed at around 9 PM. About 5 hr after the last infiltration, two systemic (upper untreated) leaves were either collected for defense gene analysis or challenge-inoculated with Psm as described below (“assessment of plant resistance to Psm”).

Biological induction of SAR. Three lower leaves on each 4-week-old soil-grown Arabidopsis plant were infiltrated from the abaxial side with 10 μM MgCl₂ (mock) or a Psm suspension (OD₅₉₀ = 0.002). The SAR activation treatment was conducted between 10 and 11 AM. Two days later, two systemic leaves were either collected for gene expression analysis or challenge-inoculated with Psm as described below (“assessment of plant resistance to Psm”).

Assessment of plant resistance to Psm. To assess Psm resistance in the NAD(P)⁺-treated leaves or the systemic leaves, a Psm suspension (OD₅₉₀ = 0.001) was infiltrated from the abaxial side into the target leaves using a 1-μL needleless syringe. A total of 12 plants were used per genotype/treatment. Two and a half to three days later, eight leaves per genotype/treatment from eight plants (one leaf from each plant) were collected to examine the growth of the pathogen. A leaf disk (~7 mm in diameter) was removed from each leaf using a hole puncher. Each leaf disk was placed into 500 μL of 10 mM MgCl₂ in a 2-μL microcentrifuge tube and ground forcefully using a plastic pestle. Twenty-fold serial dilutions of the homogenate were plated on trypticase soy agar (BD) plates supplemented with 50 μg/mL streptomycin to determine bacterial concentrations. Bacterial growth rates were expressed as colony-forming units (cfu) per leaf disk area. All experiments depicted in the figures were repeated at least three times with similar results.

RNA analysis. For reverse transcription (RT), total RNA was treated with DNase I (ThermoFisher Scientific) at 37°C for 30 min. After inactivation of the DNase I, RT was performed using SUPERSCRIPT First-strand Synthesis System (ThermoFisher Scientific) and 2 μg of the DNase-treated RNA in a 20 μL reaction. Quantitative PCR was performed using SYBR Green protocol (Applied Biosystems) with 1 μM primers and 0.2 μL aliquot of RT product in a total of 12.5 μL per reaction. Reactions were run and analyzed on an MX3000P real-time PCR machine (Stratagene) according to the manufacturer’s instructions. A standard curve was made by determining the threshold cycle (Ct) values for a dilution series of the RT reaction product for each primer pair. For each reaction, the Ct was determined by setting the threshold within the logarithmic amplification phase. The relative quantity of a gene is expressed in relation to UBQUITINS (UBQ5) using the formula 2⁻^[Ct(Gene)-Ct(UBQ5)] where 2 represents perfect PCR efficiency. All experiments described in the figures were repeated at least three times with similar results.

NAD⁺ movement assay. Three lower leaves on a 4-week-old soil-grown Arabidopsis plant or two lower leaves on a 5-week-old soil-grown N. benthamiana plant were infiltrated from the abaxial side with a water solution of 1 μL unlabeled NAD⁺ plus 6.25 mM 32P-NAD⁺ (specific activity 800 Ci/mmol; PerkinElmer, USA). The treated plants were incubated at 22–24°C under a 16-h light/8-h dark regime for 24 hr. The infiltrated leaves and two systemic leaves from each plant were collected, wrapped with plastic waterproof membrane, and exposed to X-ray film for 24 hr at ~80°C. The X-ray film was then developed to visualize the radioactive in the leaves.

Protein purification. For purification of MPB, MPB-eLexCK-VL2, and MPB-eDORN1, a single colony of BL21(DE3) carrying the corresponding plasmid was cultured overnight at 37°C (5 mL, Lysogeny broth) (LB) with 20 μg/mL ampicillin. One mL of the seed culture was added to 500 mL fresh LB medium with 50 μg/mL ampicillin and cultured at 37°C with shaking to an OD₆₀₀ of 0.4. Isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.3 mM and the culture was allowed to grow for another 16–18 hr at 18°C before the cells were harvested for protein extraction. MPB-fusion proteins were purified with amyllose resin according to the protocol supplied by the manufacturer (New England BioLabs).

NAD⁺-binding assays. Amylose resin beads with the bound proteins were suspended in binding buffer (10 mM HEPES, pH 7.5, 5 mM MgCl₂) and aliquoted in 86 μL portions for individual binding reactions. For total binding assay, 10 μL binding buffer and 4 μL 32P-NAD⁺ (6.25 μCi) were added, resulting in 250 nmol 32P-NAD⁺ in the final 100 μL reaction mixture. For nonspecific binding, 10 μL of 2.5 mM unlabeled NAD⁺ and 4 μL 32P-labeled NAD⁺ were added, resulting in 250 μmol unlabeled NAD⁺ and 250 μmol 32P-NAD⁺ in the final 100 μL reaction mixture. The mixtures were incubated for 30 min at room temperature with gentle mixing every 5 min. The beads containing the binding reactions were then precipitated by centrifugation at 500 x g for 5 min, washed three times with the binding buffer, re-suspended in 10 mL SciNiVerse BD Cocktail (ThermoFisher Scientific) per sample, and carefully transferred into scintillation vials. The vials were placed in a Beckman Coulter LS6500 Multi-Purpose Scintillation Counter (Beckman) and bound 32P-NAD⁺ was quantified by scintillation counting. Specific binding was calculated by subtracting the nonspecific binding from the total binding. For saturation binding assay, amylose resin beads with the bound proteins were incubated with different concentrations (50, 200, 500, 1000, 1500, and 2000 μM) of 32P-NAD⁺ in the absence (for total binding) or presence (for nonspecific binding) of additional 1000-fold unlabeled NAD⁺ in the binding buffer. The association constant (Kₘ) was calculated by one site specific saturation model using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). For competitive binding assay, amylose resin beads with the bound proteins were incubated with 250 nmol 32P-labeled NAD⁺ in the presence of different concentrations (100 nM, 1 μM, 10 μM, 100 μM, and 1 mM) of unlabeled nucleotides (NAD⁺, NADP⁺, ATP, and ATP) in the binding buffer. The 50% inhibition concentration (IC₅₀) values were calculated by importing the data points into GraphPad Prism 7 using the one site Fit logIC₅₀ competition model.

For microsome-based binding assays, Arabidopsis plants were grown under a 15-hr-light/9-hr-dark regime for about 6 weeks. Rosette leaf tissues were homogenized with a mortar and chilled in 1 mL 1× cold binding buffer, and were quantified by scintillation counting.

Microarray analysis. Three lower leaves on each four-week-old soil-grown plant were infiltrated with either 10 μM MgCl₂ (−SAR) or Psm (OD₅₉₀ = 0.002) (+SAR). Two days later, the systemic leaves were collected for total RNA extraction. Total RNA samples were subjected to microarray analysis using the Affymetrix microarray platform, which was performed at Interdisciplinary Center for Biotechnology Research Gene Expression and Genotyping Core at The University of Florida. RNA concentration was determined with a NanoDrop Spectrophotometer (ThermoFisher Scientific) and sample quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). All microarray sample preparation used the GeneChip 3′ IVT Plus Express kit (ThermoFisher Scientific), and reactions were done following the manufacturer’s protocols. In brief, cDNA was synthesized from 500 ng of total RNA using IVT kit, then labeled using IVT kit. Modified nucleotide was incorporated. The biotin-labeled cRNA (amplified RNA) was then purified and fragmented. Eleven μg of biotin-labeled cRNAs were hybridized with rotation at 45°C for 16 hr to the GeneChip Arabidopsis Genome ATH1 Array (ThermoFisher Scientific). The arrays were washed and stained with the reagents supplied in GeneChip Hybridization Wash and Stain kit (ThermoFisher Scientific) on an Affymetrix Fluidics Station 450, and scanned with a GeneChip 7 G Scanner (ThermoFisher Scientific).

The microarray data were pre-processed and normalized using the affy package 2. The Robust Multichip Analysis approach was applied for the normalization. After normalization, the empirical Bayes moderated t-statistics, which is implemented in the limma Bioconductor package 27, was performed for differential expression detection. In each comparison, a p value and fold change
were computed for each gene locus. The gene expression fold changes were computed based on the normalized log-transformed signal intensity data. To control hypothesis and correct multiple testing, the value of q was calculated and used to assess the significance of each test using Benjamini and Hochberg’s approach. Genes with an absolute fold change ≥ 2 and a q value ≤ 0.05 were considered as significantly induced or suppressed and were further explored to obtain overlapped genes among genotypes. The vein function implement in limma package was applied to compute classification counts and draw a vein diagram.

**Plasmid construction and plant transformation.** For expression of the MBP-LecRK-VI fusion protein in *E. coli*, the DNA fragment encoding the extracellular domain (AAs 23-310) of LecRK-VI.2 was amplified using the primers XbaI-LecRK-VI.2 P and HindIII-LecRK-VI.2 R (all primers are listed Supplementary Table 1). The PCR products were digested with XbaI and HindIII and cloned into the XbaI/HindIII site of pMAL-p2X, generating pMAL-p2X-LecRK-VI.2. The pMAL-p2X-EDORNI plasmid has previously been described.

For the expression of the MBP-LecRK-VL2KD (AAs 331–682) and GST-BAK1KD (AAs 250–615) fusion proteins in *E. coli*, DNA fragments encoding the KDs of LecRK-VL2 and BAK1 were amplified using primers listed in Supplementary Table 1. The presence of the expected mutations in the resulting constructs was verified by DNA sequencing. The plasmids pCambia1300S-LecRK-VL2-FLAG and pCambia1300S-BAK1-FLAG, respectively. For transient expression in *N. benthamiana*, the coding regions of LecRK-VL2 and BAK1 were amplified using primers LecRK-VL2-XbaI-KDR and BAK1-XbaI-KDR and cloned into the XbaI/Sall site of pCAMBIA1300S-FLAG and the Sall/Cit site of pCAMBIA1300S-GFP, respectively. For generation of transgenic Arabidopsis plants expressing LecRK-VL2-HA, the DNA fragment containing the LecRK-VL2 promoter and coding region was amplified using primers XbaI-LecRK-VL2PF and BglII-3'HA-lecRK-VL2 R and cloned into the BamHI/Sall site of pCAMBIA1300S-GFP, resulting in pCambia1300S-LecRK-VL2-FLAG and pCambia1300S-BAK1-GFP, respectively. For generation of transgenic Arabidopsis plants expressing LecRK-VL2-HA, the DNA fragment containing the LecRK-VL2 promoter and coding region was amplified using primers XbaI-LecRK-VL2PF and BglII-3'HA-lecRK-VL2 R and cloned into the BamHI/Sall site of pCB302, resulting in pCB302-LecRK-VL2-LecRK-VL2-HA. Site-directed mutagenesis of Asp494 of LecRK-VI.2 and Lys317 of BAK1 was performed using primers LecRK-VI.2KD and BAK1-XbaI-KDR and cloned into the BamHI/Sall site of pCB302, resulting in pCB302-LecRK-VL2-LecRK-VL2-HA.

**Pull-down and co-immunoprecipitation assays.** One microgram MBP or MBP-LecRK-VL2KD was incubated with 1 μg GST-BAK1KD in a binding buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.05% IGEPAL CA-630, and 1% [v/v] protease inhibitor cocktail) at 2 mL/g tissue powder. Samples were clarified by centrifugation at 16,000 × g at 4 °C for 20 min. For *N. benthamiana*, the supernatant was incubated with anti-GST (Cell Signaling, 2625 S, dilution 1:1000) antibodies to detect immunoprecipitated GST-BAK1KD, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. Plant Cell 24, 5123–5141 (2011).

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Acknowledgements

We thank Dr. Ping He (Texas A&M University, USA) for baki-5, bkk1, baki-5 bkk1, bkk1, and BAK1/BAKI-GFP seeds, Dr. Sheng Yang He (Michigan State University, USA)
for fls2 efr seeds, Dr. Jean Greenberg (University of Chicago, USA) for ald1 and azl1 seeds, Dr. Yusuke Saijo (Nara Institute of Science and Technology, Japan) for pepr1 pepr2 seeds, Dr. Jayoti Shah (University of North Texas, USA) for gdl1 seeds, and Dr. Cyril Zipfel for protocols for measuring ROS burst from leaf disks. This work was partially supported by a Research Opportunity Seed Fund grant from the University of Florida Office of Research awarded to Z.M. and a grant from the National Science Foundation (ISO-1758932) awarded to Z.M.

**Author contributions**
C. Wang and Q. Li performed the gene expression, resistance assessment, protein–protein association, and site-directed mutagenesis experiments. X. Huang and Q. Li carried out the radioactivity experiments. Y.Z. conducted the microarray experiment. J.-L.L. analyzed the microarray data. Z.M. secured funding, conceived the research, and wrote the manuscript.

**Competing interests**
The authors declare no competing interests.

**Supplementary information**
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-12781-7.

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**Peer review information** Nature Communications thanks Robin Cameron, Stephen Chivasa, Jean Greenberg and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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