Molecular Plant
Research Report

CellPress
Partner Journal

A karyopherin constrains nuclear activity of the NLR protein SNC1 and is essential to prevent autoimmunity in *Arabidopsis*

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https://doi.org/10.1016/j.molp.2021.06.011

ABSTRACT

The nucleotide-binding and leucine-rich repeat (NLR) proteins comprise a major class of intracellular immune receptors that are capable of detecting pathogen-derived molecules and activating immunity and cell death in plants. The activity of some NLRs, particularly the Toll-like/interleukin-1 receptor (TIR) type, is highly correlated with their nucleocytoplasmic distribution. However, whether and how the nucleocytoplasmic homeostasis of NLRs is coordinated through a bidirectional nuclear shuttling mechanism remains unclear. Here, we identified a nuclear transport receptor, KA120, which is capable of affecting the nucleocytoplasmic distribution of an NLR protein and is essential in preventing its autoactivation. We showed that the *ka120* mutant displays an autoimmune phenotype and NLR-induced transcriptome features. Through a targeted genetic screen using an artificial NLR microRNA library, we identified the TIR-NLR gene *SNC1* as a genetic interactor of *KA120*. Loss-of-function *snc1* mutations as well as compromising SNC1 protein activities all substantially suppressed *ka120*-induced autoimmune activation, and the enhanced SNC1 activity upon loss of *KA120* functionappeared to occur at the protein level. Overexpression of *KA120* efficiently repressed SNC1 activity and led to a nearly complete suppression of the autoimmune phenotype caused by the gain-of-function *snc1-1* mutation or *SNC1* overexpression in transgenic plants. Further florescence imaging analysis indicated that SNC1 undergoes altered nucleocytoplasmic distribution with significantly reduced nuclear signal when KA120 is constitutively expressed, supporting a role of KA120 in coordinating SNC1 nuclear abundance and activity. Consistently, compromising the SNC1 nuclear level by disrupting the nuclear pore complex could also partially rescue *ka120*-induced autoimmunity. Collectively, our study demonstrates that KA120 is essential to avoid autoimmune activation in the absence of pathogens and is required to constrain the nuclear activity of SNC1, possibly through coordinating SNC1 nucleocytoplasmic homeostasis as a potential mechanism.

Key words: karyopherin, KA120, SNC1, nucleocytoplasmic transport, immune activation

Jia M., Shen X., Tang Y., Shi X., and Gu Y. (2021). A karyopherin constrains nuclear activity of the NLR protein SNC1 and is essential to prevent autoimmunity in *Arabidopsis*. Mol. Plant. **14**, 1733–1744.

INTRODUCTION

Both plants and animals evolved nucleotide-binding (NB), leucine-rich repeat (LRR) proteins (NLR proteins) as intracellular immune receptors to detect microbial ligands and coordinate immune activation (Jones et al., 2016). Plant NLRs can be further divided into two major classes according to their N-terminal signaling domains, namely Toll-like/interleukin-1 receptor (TIR) domain-containing NLR (TNL) and coiled-coil domain-containing NLR (CNL) (Jubic et al., 2019; Zhou and Zhang, 2020). Plant NLRs perceive a variety of pathogen-secreted virulent proteins called effectors and subsequently activate effector-triggered immunity (ETI) (Chisholm et al., 2006; Jones and Dangl, 2006; Dodds and Rathjen, 2010; Maekawa et al., 2011; Spoel and Dong, 2012). ETI activation results in permeabilization of the nuclear pore complex (NPC) and

Published by the Molecular Plant Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and CEMPS, CAS.

massive nuclear shuttling of signaling molecules (Garcia and Parker, 2009; Wang et al., 2014; Cui et al., 2016; Gu et al., 2016), which leads to global transcriptome reprogramming and robust activation of defense gene expression (Tsuda et al., 2009; Buscaill and Rivas, 2014). ETI is usually accompanied by a localized programmed cell death event termed hypersensitive response (HR). HR may be promoted directly by the activation of some CNLs, which can form an oligomeric complex called resistosome and translocate to the plasma membrane, where they are capable of forming calcium permeable channel to trigger immunity and cell death (Wang et al., 2019a, 2019b; Bi et al., 2021). TNL-dependent HR requires NAD^+ cleavage by the TIR domain, which appears to be a conserved mechanism for cell death signaling in both plant and animal cells (Horsefield et al., 2019; Wan et al., 2019; Duxbury et al., 2020). Local ETI activation also induces systemic acquired resistance (SAR) that primes distal, uninfected tissues to confer enhanced resistance against subsequent pathogen attacks (Spoel and Dong, 2012).

Due to the profound consequence and considerable fitness cost of ETI activation, the cellular concentration and activity of NLRs must be tightly controlled in the absence of pathogens to prevent autoactivation. On the other hand, proper levels of NLRs are required for pathogen detection and subsequent immune signaling (Bieri et al., 2004; Holt et al., 2005). Thus, plants have evolved a multiplicity of regulatory mechanisms to carefully maintain the homeostasis of NLRs (Shirasu, 2009; Li et al., 2015; Lai and Eulgem, 2018).

At the transcription level, the steady-state NLR expression is regulated epigenetically by DNA methylation and histone modifications (Li et al., 2010b; Palma et al., 2010; Xia et al., 2013; Yu et al., 2013; Zou et al., 2014). After transcription, some NLR transcripts are targeted by microRNAs, which induce phased secondary small interfering RNAs (phasiRNAs) that repress the expression of a wide set of NLRs (Yi and Richards, 2007; Zhai et al., 2011; Li et al., 2012; Shivaprasad et al., 2012; Boccara et al., 2014; Zhang et al., 2016). In *Arabidopsis*, this global suppression of NLRs can be relieved upon activation of the TNL SNC1 to facilitate ETI induction (Cai et al., 2018). Another post-transcriptional regulation of NLRs involves alternative splicing (AS) events, which are either constitutive or responsive to defense signaling. Many AS transcripts of NLRs have been shown to be essential for successful immune activation (Dinesh-Kumar and Baker, 2000; Zhang and Gassmann, 2003; Tang et al., 2013). In *Arabidopsis*, the cyclin L homolog MOS12, the conserved spliceosome-associated MOS4-associated complex, RNA-binding protein MOS2, and the Transportin-SR MOS14 have been reported to play a role in proper splicing of NLRs (Xu et al., 2011, 2012; Copeland et al., 2013). At the post-translational level, molecular chaperone HSP90, SGT1, and RAR1 are required for the proper folding and accumulation of some NLRs, which are necessary for ETI induction (Hubert et al., 2003; Bieri et al., 2004; Holt et al., 2005; Li et al., 2010a). Meanwhile, the protein abundance of some NLRs, including SNC1 and RPS2, have been shown to be negatively regulated by components in the ubiquitin-proteasome pathway, including Constitutive expresser of PR genes 1 (CPR1), an F-box protein that is part of the SCF protein degradation complex (Cheng et al., 2011; Gou et al., 2012), and Mutant, *snc1*-enhancing 3 (MUSE3), an E4 ligase that mediates polyubiquitination of proteins (Huang et al., 2014). These proteins are required for preventing autoimmune induction caused by overaccumulation of NLR proteins.

Nucleocytoplasmic partitioning constitutes another critical layer of spatial and temporal regulation on the activity of many NLRs, particularly TNLs (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007; Caplan et al., 2008; Zhu et al., 2010b; Padmanabhan et al., 2013). Many TNLs undergo nuclear translocation for signaling, and their nucleocytoplasmic distribution ratio is tightly controlled and can directly influence the defense activation (Garcia and Parker, 2009; Cui et al., 2015). For example, enhanced nuclear accumulation of SNC1 results in autoimmunity (Mang et al., 2012; Xu et al., 2014), and fusion of SNC1 to nuclear export signal efficiently disrupts SNC1-induced immune activation, suggesting that the nuclear pool of SNC1 is essential for defense signaling but also needs to be tightly controlled (Palma et al., 2005; Cheng et al., 2009; Zhu et al., 2010a, 2010b; Mang et al., 2012). SNC1 was predicted to possess a nuclear localization signal, and its nuclear accumulation requires the intact NPC and perhaps also functional importin-α3 (Palma et al., 2005; Cheng et al., 2009; Ludke et al., 2020). However, whether and how the nucleocytoplasmic distribution of NLRs is regulated by a bidirectional nuclear shuttling mechanism to coordinate their nuclear activities is still largely unclear.

Here, we report a nuclear transport receptor, KA120, which is essential to constrain SNC1 nuclear activity. Loss of KA120 led to autoactivation of immune responses that mimic NLR activation, which can be largely suppressed by *pad4* and *eds1* mutation or repressing the SNC1 activity at either the post-transcriptional or post-translational level. Constitutive expression of KA120 efficiently suppressed SNC1 activity and resulted in an almost complete rescue of the autoimmune phenotype in the *snc1-1* autoactive mutant as well as SNC1-overexpression plants. At the cellular level, we found that overexpression of *KA120* was capable of shifting the nucleocytoplasmic distribution of SNC1 by significantly reducing its nuclear retention in both protoplast transient assays and in transgenic plants. Consistently, compromising SNC1 nuclear level by disrupting the NPC alleviated the *ka120*-induced autoimmunity. These observed effects appear to be specific to KA120 among *Arabidopsis* karyopherin-β family proteins, suggesting a specialized role of KA120 in regulating SNC1 nuclear activities.

RESULTS AND DISCUSSION

Identification of *KA120* as a potential immune regulator

Previously, we reported a critical role of an exportin (XPO4) in negatively regulating *cpr5*-dependent immune activation (Xu et al., 2021). To systematically investigate the regulatory role of nuclear transport receptors in plant immune responses, we collected T-DNA insertion mutant lines for all 18 karyopherin-β genes in *Arabidopsis*, including importins and exportins (Figure 1A). Among them, two independent mutant lines for the *KA120* gene (AT3G08960) displayed similar phenotypes reminiscent of constitutive immune activation, including dwarf stature and wrinkled leaves (Figure 1B and Supplemental Figure 1A). We designated the two mutant lines as *ka120-1* (Salk_148803) and *ka120-2* (GABI-605D06). The *ka120* mutants are also late flowering (Figure 1C) and compromised in seed

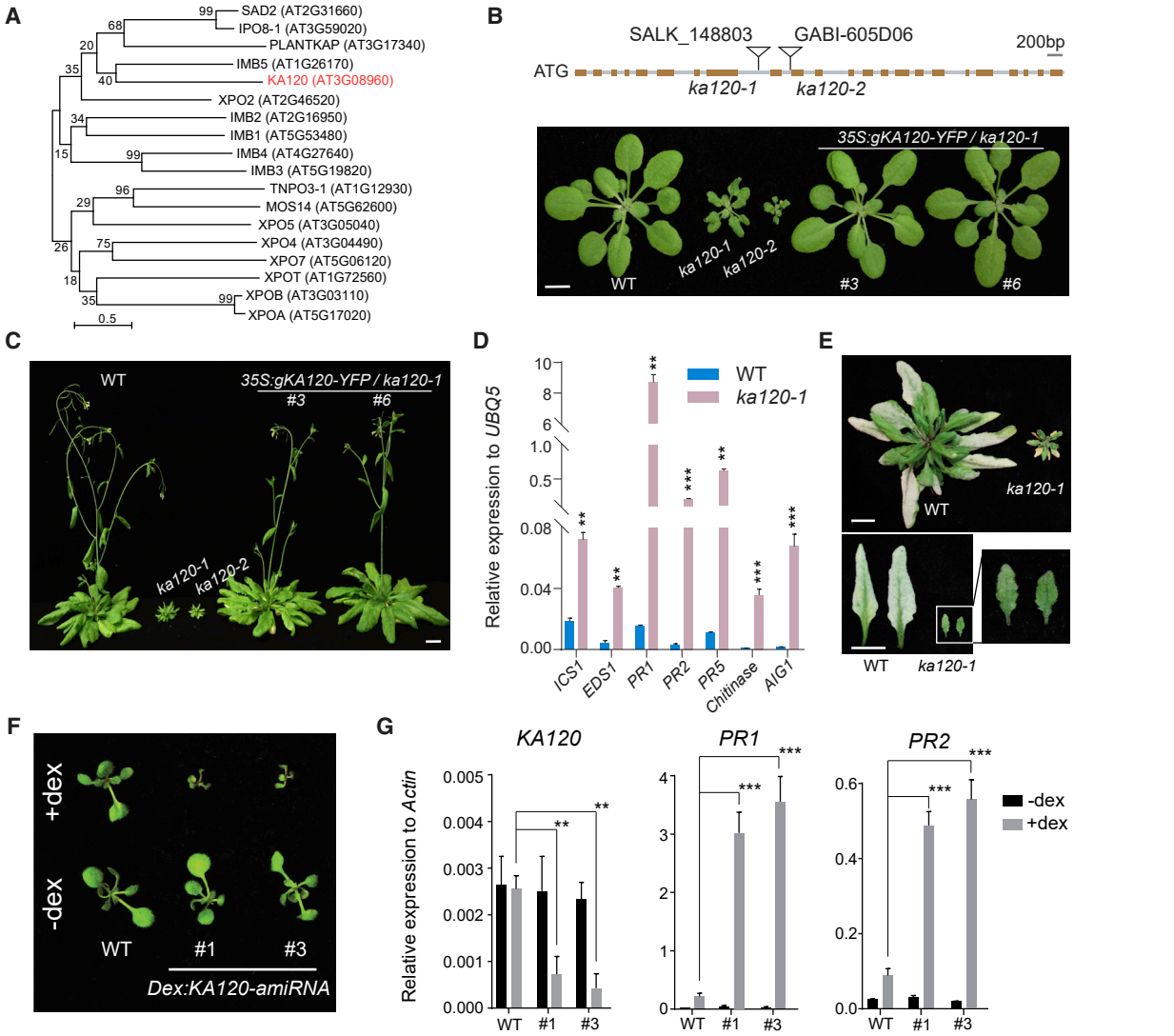

Figure 1. *KA120* is a critical negative regulator of immune induction.

(A) Phylogenetic analysis of *Arabidopsis* karyopherin-β family using protein sequences and maximum-likelihood method with 1000 bootstraps. Neighbor-joining tree was constructed using MEGAX 10.1.8. Scale bar represents amino acid substitutions per position.

(B and C) Four-week-old **(B)** and 6-week-old **(C)** plants of Col-0 (WT), *ka120-1*, *ka120-2*, and two independent lines of *35S:gKA120-YFP* in the *ka120-1* background (T3 generation). The gene structure of At*KA120* and the T-DNA insertion sites are shown in the upper panel of **(B)**. Scale bars, 1 cm.

(D) Relative expression levels of defense marker genes to *UBQ5* in WT and the *ka120-1* plants measured by qRT–PCR. Data are presented as mean ± SD ($n = 2$ technical replicates). Student's *t*-tests were performed for each gene: $^{**}p < 0.01$, $^{***}p < 0.001$. Similar results were obtained twice.

(E) Four-week-old WT and *ka120-1* plants were inoculated with the powdery mildew pathogen *Golovinomyces cichoracearum*, and images were taken 6 days post inoculation. The lower panel shows infected leaves of similar age from WT and *ka120-1* plants. Scale bar, 1 cm.

(F) One-week-old *Dex:KA120-amiRNA* transgenic plants grown on half-strength MS agar plates in the presence (+) or absence (−) of 5 μM dexamethasone (dex). Results from two independent lines and WT control are shown.

(G) Relative expression levels of *KA120*, *PR1*, and *PR2* to *Actin* at 24 h after 25 μM dex application to leaves of 3-week-old soil-grown WT and *Dex:KA120-amiRNA* transgenic plants. qRT–PCR data are presented as mean ± SD ($n = 3$ biological replicates). Student's *t*-tests were performed: $^{**}p < 0.01$, $^{***}p < 0.001$.

production. These observed phenotypes can be fully complemented by a translational fusion of *KA120* genomic DNA to YFP (*35S:gKA120-YFP*) in *the ka120-1* background (Figure 1B and 1C; Supplemental Figure 1A), suggesting that compromised KA120 function is responsible for the observed mutant defects. The *ka120* phenotype appears to be unique among karyopherin-β genes because T-DNA insertion mutants of *KA120*'s close homologs, including *IMB5*, *PLANTKAP*, *IP O 8-1*, and *SAD2*, did not show a similar autoimmune phenotype (Supplemental Figure 1B and 1C), implying functional specificity of *KA120*.

Loss of *KA120* leads to autoimmune induction

To confirm that loss of *KA120* activated immune responses, we first performed quantitative RT–PCR (qRT–PCR) and found that various defense marker genes were highly induced in the

ka120-1 mutant (Figure 1D). We then inoculated *ka120* plants with the powdery mildew pathogen *Golovinomyces cichoracearum* and observed significantly less pathogen growth compared with Columbia-0 (Col-0) wild-type (WT) plants (Figure 1E). To demonstrate a more direct role of *KA120* in immune regulation, we generated a dexamethasone (dex)-inducible artificial microRNA line (*Dex*:*KA120-amiRNA*) in the WT background. Growth of the transgenic lines on Murashige and Skoog (MS) agar plates supplied with 5 μM dex led to severe growth inhibition (Figure 1F). Transient knockdown of *KA120* expression in soil-grown plants by spraying 25 μM dex induced *PR* gene expression within 24 h (Figure 1G). These findings suggest that loss of *KA120* can lead to autoimmune induction and that *KA120* is a critical negative regulator of plant immunity.

NLR-mediated immunity is activated in the *ka120* mutant

To better understand the *ka120*-activated immune response, we performed whole-genome transcriptome profiling using 3-week-old WT and *ka120* mutant plants (GEO: GSE147683). We identified 1689 differentially expressed genes (DEGs) ($p < 0.05$, fold change >2) in the *ka120* mutant compared with WT (Figure 2A and Supplemental Table 1). Gene ontology (GO) analysis indicates that defense- and cell death-related genes are highly enriched in *ka120*-dependent upregulated DEGs (Figure 2B). When compared with transcriptome changes induced by RPS4, a TNL (Heidrich et al., 2013), and elf18 (Pajerowska-Mukhtar et al., 2012), a pathogen-associated molecular pattern, we found that *ka120*-dependent responses showed a predominant overlap (1166/1689, 69%) with RPS4-induced responses but considerably less overlap (446/1689, 26%) with elf18-induced responses (Figure 2C). Moreover, *ka120*-induced DEGs contain 809 RPS4-specific DEGs but only 89 elf18-specific DEGs. *ka120*-dependent DEGs also displayed a highly concordant expression pattern with temperature-dependent RPS4-triggered response (Figure 2D). These analyses suggest that NLR-activated transcriptome reprogramming is at least partially activated in the *ka120* mutant.

Consistent with the transcriptome analysis, mutations in *EDS1* and *PAD4*, which are required for immune signaling induced by some TNLs, substantially suppressed the *ka120* phenotype, including the stunted growth and late flowering (Figure 2E and 2G; Supplemental Figure 2). However, a mutation in *NDR1*, which is required for immune signaling induced by a subset of CNLs, conferred limited suppression on *ka120* phenotypes. Further RNA sequencing (RNA-seq) analysis (GEO: GSE147682) revealed that the global suppression of *ka120*-induced DEGs is significant by *pad4* but not by *ndr1* (Figure 2H), supporting our genetic analysis.

In addition to NLR-mediated immunity, we also tested the contribution of other types of defense signaling to immune activation in *ka120* plants. We found that a mutation in *NPR1* that largely abolishes SAR (Cao et al., 1997) and a mutation in *EDS5* that blocks pathogen-induced SA transport and accumulation (Serrano et al., 2013) had little effect on the *ka120* phenotype (Figure 2F and 2G; Supplemental Figure 2A). These observations strongly support a hypothesis that *EDS1*/*PAD4*-dependent NLR-mediated immune signaling is activated in *the ka120* mutant. However, it is worth noting that the suppression of *ka120*-induced stunted growth and defense gene upregulation by *pad4* is not complete (Figure 2G and 2H), suggesting that additional defense signaling may be activated in the *ka120* mutant.

An artificial NLR microRNA library-based genetic screen for *ka120* suppressors identified *SNC1*

To determine whether the NLR-mediated immune activation in *the ka120* mutant is due to the activation of NLR receptors and if so, which NLR(s) is activated, we designed an artificial microRNA (amiRNA) library to systematically disrupt the expression of NLR genes in the *ka120-1* mutant (Figure 3A). A total of 116 amiRNAs targeting 108 *Arabidopsis* NLR genes (73 TNLs and 35 CNLs) were designed (Supplemental Table 2), with some amiRNAs targeting several closely related NLRs. These amiRNAs were individually cloned into a binary vector, and their expression was driven by a 35S CaMV constitutive promoter. We then pooled an equal amount of these amiRNA vectors and bulk transformed the library into *ka120* heterozygous plants using agrobacteria-mediated transformation. In the T1 generation, we genotyped to obtain *ka120* homozygous T1 transformants following Basta selection. In total, 262 *ka120* homozygous T1 transformants were obtained. Under the premise that all the amiRNA constructs have been successfully transformed and each transformant carries at least one amiRNA construct, our screen is expected to have covered 89% of the amiRNAs in theory (Figure 3B). However, it is more likely that the amiRNA coverage is lower due to possible incomplete transformation of the library. Out of the 262 transformants, three independent lines exhibited observable suppression of the stunted growth and wrinkled leaf phenotype in the *ka120* mutant (Supplemental Figure 3A). Subsequent DNA extraction and sequencing showed that all three lines contained an amiRNA construct that specifically targets a single TNL gene, *SNC1* (Supplemental Figure 3B).

To validate this suppression, we crossed the loss-of-function *SNC1* mutant *snc1-r1* with *ka120-1*. The *ka120-1 snc1-r1* double mutant significantly suppressed stunted growth in the *ka120* mutant, to a similar extent to *ka120-1 pad4* (Figure 3C and 3D). These results suggest that *SNC1* plays a critical role in *ka120*-dependent immune induction.

SNC1 is autoactivated at the protein level upon loss of *KA120* function

To further investigate the mechanism of *SNC1*-dependent autoimmune induction in *ka120*, we first analyzed the transcription level of *SNC1* in the *ka120* mutant. Data retrieved from two independent transcriptome profiling results indicate that the *SNC1* expression did not appear to be altered significantly in the *ka120* mutant compared with WT in 3-week-old plants (Figure 3E). However, RNA-seq analysis revealed that the expression of at least 50 other NLR genes are significantly upregulated in the *ka120* mutant compared with WT (Figure 3F and Supplemental Table 3), consistent with a previous report that activation of SNC1 relieves the global repression of NLR gene expression (Cai et al., 2018). In older plants (5 weeks old), the *SNC1* transcript level is elevated significantly in the *ka120* background compared with WT; however, this elevation can be completely suppressed in the *ka120 pad4* and *ka120 eds1* double mutants (Supplemental Figure 3C), suggesting that this elevation is a secondary effect and is likely caused by defense amplification induced by SNC1 activation. Thus, we conclude

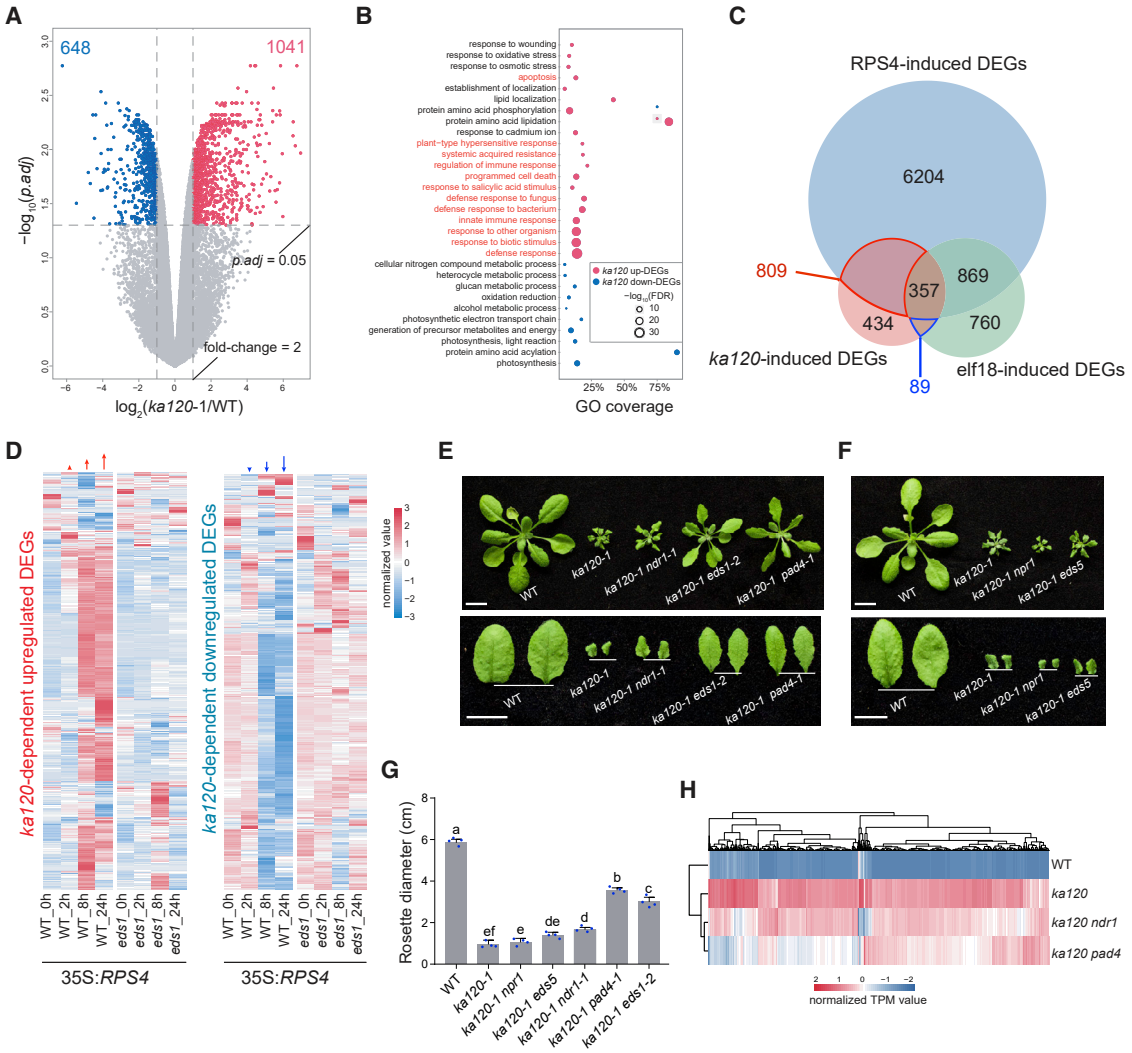

Figure 2. NLR-mediated immunity is activated in the *ka120* mutant.

(A) Volcano plot showing differentially expressed genes (DEGs) ($p < 0.05$ and fold change >2) in the *ka120-1* mutant compared with WT determined by microarray analysis (GEO: GSE147683). Up- and downregulated DEGs are displayed as red and blue dots, respectively. The entire rosette of 3-week-old plants were sampled, and three biological replicates were used for the analysis.

(B) Gene ontology (GO) term analyses of *ka120*-dependent DEGs (red dots for upregulated DEGs and blue dots for downregulated DEGs). Defense- and cell death-related GO terms are labeled in red.

(C) Comparative analyses of *ka120* microarray data with defense-related microarray datasets (RPS4, GEO: GSE50019 and elf18, GEO: GSE34047). Venn diagram shows 809 and 89 overlapping genes between *ka120*-induced DEGs and RPS4- and elf18-specific DEGs, respectively ($p < 0.05$ and fold change >2).

(D) Expression patterns of the *ka120*-dependent up- and downregulated DEGs during temperature-controlled RPS4 activation in WT and *eds1* background (GEO: GSE50019). Increased arrow length indicates increased number of *ka120*-induced DEGs that show concordant upregulated (red) or downregulated (blue) expression pattern with RPS4-induced response.

(E and F) Rosettes and the third and fourth leaves of 4-week-old *Arabidopsis* plants with indicated genetic background. Scale bars, 1 cm.

(G) Statistics of the rosette diameter of 4-week-old *Arabidopsis* plants. Data are presented as mean ± SD ($n = 4$). Data points are shown as blue dots. Tukey's HSD test was performed.

(H) Heatmap showing the expression of *ka120*-induced upregulated DEGs ($p < 0.05$ and fold change >2) in WT, *ka120-1*, *ka120-1 ndr1*, and *ka120-1 pad4* plants determined by RNA-seq analysis (GEO: GSE147682). The entire rosette of 3-week-old plants were sampled, and three biological replicates were used for analysis.

that activation of SNC1 in the *ka120* mutant is not initiated by an upregulation in *SNC1* transcription.

Next, we grew *ka120* mutant plants under 28°C, a condition that compromises SNC1 protein activity (Yang and Hua, 2004). We found that the *ka120* phenotype can be partially rescued (Figure 3G). We also generated *ka120* transgenic lines that overexpressed a dominant-negative form of SNC1 (SNC1DN), which contains mutations in the SNC1 P-loop motif for ATP binding (Xu et al., 2014), and the *ka120* phenotype was also alleviated (Figure 3H). Furthermore, we overexpressed *CPR1*, an F-box protein that is part of the SCF complex, which has been shown

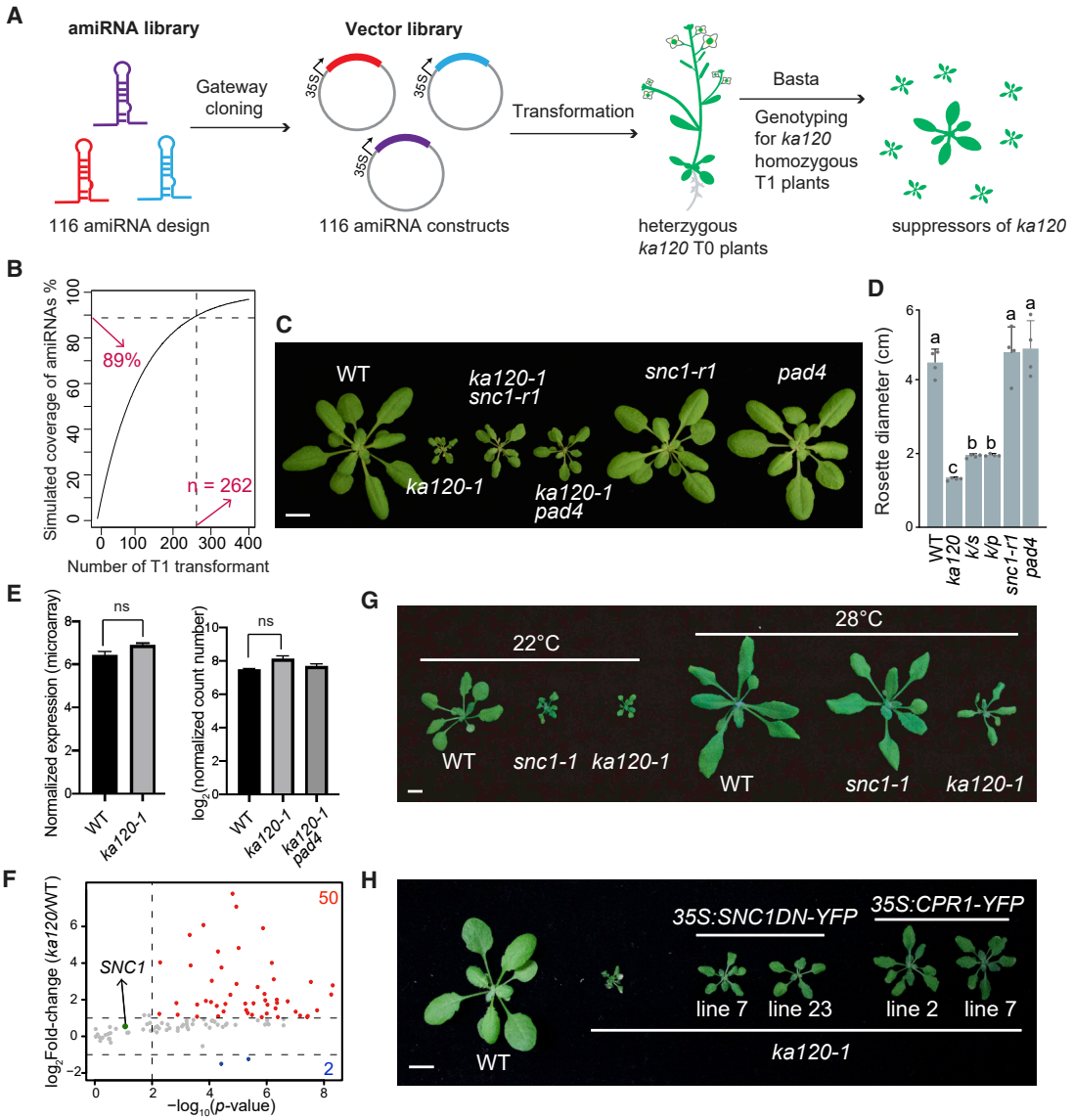

Figure 3. SNC1 is activated at the protein level upon loss of *KA120* function.

(A) Schematic for the NLR artificial microRNA (amiRNA) library-based targeted genetic screen for *ka120* suppressors. One hundred and sixteen amiRNAs targeting 108 *Arabidopsis* NLR genes were cloned individually into an expression vector driven by the 35S CaMV promoter. An equal amount of the 116 amiRNA constructs were pooled, and the resulting vector library was bulk transformed into a *ka120-1* heterozygous background. Following Basta screen in soil, T1 progenies were PCR-genotyped to select *ka120-1* homozygous plants, and suppressors were identified based on the recovery of the stunted growth and wrinkled leaves.

(B) Simulated relationship between the number of T1 transformants obtained and the coverage of amiRNAs in the library, assuming that all amiRNA constructs being transformed into T0 plants. The simulation process is based on the multinomial distribution in the probability theory and realized by Python. The simulation result was plotted by R.

(C) Suppression of *ka120* stunted growth phenotype by the loss-of-function *snc1-r1* mutant. Four-week-old plants are shown. Scale bar, 1 cm.

(D) Statistics of the rosette diameter of 4-week-old WT, *ka120-1*, *ka120-1 snc1-r1* (k/s), *ka120-1 pad4* (k/p), *snc1-r1*, and *pad4* plants. Data are presented as mean ± SD (n = 4). Data points are shown as gray dots. Tukey's HSD test was performed.

(E) Transcription level of *SNC1* in indicated genetic backgrounds. Data were retrieved from microarray (left, GEO: GSE147683) and RNA-seq (right, GEO: GSE147682) analyses (n = 3 biological replicates). Student's t-tests were performed.

(F) Expression level of NLR genes in the *ka120-1* mutant compared with WT measured by RNA-seq. The 50 significantly upregulated NLR genes ($p < 0.01$ and fold change >2) are labeled by red dots. *SNC1* is labeled by the green dot.

(G) Three-week-old *Arabidopsis* plants grown under 22°C and 28°C. Scale bar, 1 cm.

(H) Suppression of *ka120* phenotype by overexpressing YFP-tagged dominant-negative SNC1 (SNC1DN) and CPR1 protein. Three-week-old T3 transgenic plants are shown. Scale bar, 1 cm.

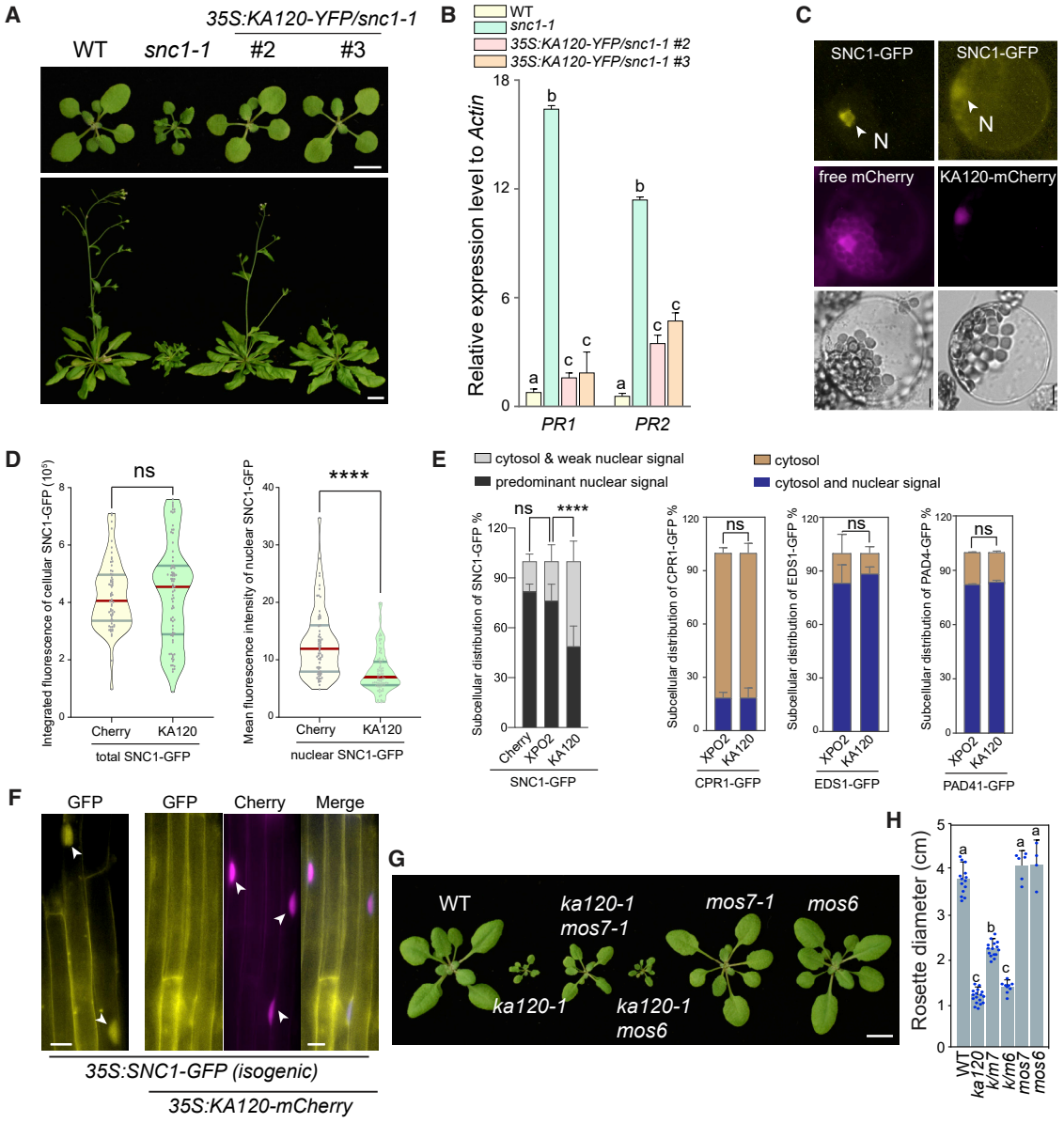

Figure 4. KA120 specifically affects the nucleocytoplasmic homeostasis and activity of SNC1.

(A) Suppression of the *snc1-1* autoimmune phenotype by overexpressing KA120-YFP in two independent transgenic lines. Three-week-old and 6-week-old *Arabidopsis* plants are shown. Scale bars, 1 cm.

(B) Suppression of defense gene expression in the *snc1-1* mutant by overexpressing *KA120*. qRT–PCR was performed to measure the relative expression level of *PR1* and *PR2* to *Actin* (n = 3 biological replicates). Tukey's HSD test was performed.

(C) Transient coexpression of SNC1-GFP with free mCherry or KA120-mCherry in *Arabidopsis* protoplasts. Arrowheads indicate the nucleus. Scale bars, 10 μm.

(D) Violin plots showing the integrated (total) fluorescence of SNC1-GFP in the whole cell and the mean fluorescence intensity of nuclear SNC1-GFP (nucleus integrated fluorescence/nucleus area) for samples coexpressing SNC1-GFP with free mCherry (n = 63) or KA120-mCherry (n = 80). The first and third quantiles are labeled in blue and the medians are labeled in red. Student's t-tests were performed: $^{****}p < 0.0001$; ns, not significant.

(E) Categorical analyses of subcellular distribution of SNC1/CPR1/EDS1/PAD4 fused to GFP upon transient expression in *Arabidopsis* protoplasts. The percentage of protoplasts falling into the different categories was quantified with samples coexpressing GFP-fused SNC1/CPR1/EDS1/PAD4 and free mCherry/XPO2-mCherry/KA120-mCherry. SNC1+mCherry (n_1 = 120, n_2 = 33), SNC1+XPO2-mCherry (n_1 = 126, n_2 = 407), SNC1+KA120-mCherry (n_1 = 160, n_2 = 437), CPR1+XPO2 (n_1 = 126, n_2 = 82), CPR1+KA120 (n_1 = 67, n_2 = 107), EDS1+XPO2 (n_1 = 26, n_2 = 126), EDS1+KA120 (n_1 = 43, n_2 = 101), PAD4+XPO2 (n_1 = 91, n_2 = 79), PAD4+KA120 (n_1 = 242, n_2 = 85). n_1 and n_2 are the number of protoplasts expressing both proteins quantified in two different experiments. Fisher's exact tests were performed: $^{****}p < 0.0001$; ns, not significant.

(F) Subcellular localization of SNC1-GFP in isogenic *35S:SNC1-GFP* line with or without constitutive KA120-mCherry expression. Nuclei are labeled with arrowheads. Scale bars, 10 μm.

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to mediate degradation of multiple NLR receptors, including SNC1 (Cheng et al., 2011; Gou et al., 2012). *CPR1* overexpression partially suppressed the growth inhibition in *ka120* plants to a similar or slightly better extent than *SNC1DN* overexpression (Figure 3H). Note that in all cases, reducing SNC1 activity cannot suppress the *ka120* phenotype completely, similar to the situation in the *ka120 pad4* double mutant, suggesting that immune activation in *ka120* also involves other factors. Together, these results suggest that SNC1 is activated at the protein level in the absence of KA120, which contributes to the autoimmune induction in the *ka120* mutant.

Overexpression of *KA120* substantially suppresses SNC1 activity

To further investigate the role of KA120 in regulating SNC1 activity, we tested whether overexpression of *KA120* can suppress SNC1 activity. We generated a *35S*: *SNC1-GFP* transgenic line that displays an autoimmune phenotype. We then crossed it with a *35S*: *KA120-mCherry* line. Remarkably, in the F3 population with non-segregating *SNC1-GFP* but segregating *KA120-mCherry*, we found that the *35S*: *SNC1*-dependent autoimmune phenotype was also segregating, and a clear suppression of the autoimmune phenotype was strictly linked to the presence of *KA120-mCherry* (Supplemental Figure 4A).

To confirm this result in a cleaner system with more consistent SNC1 activity, we transformed a *35S*: *KA120-YFP* construct in the *snc1-1* background (Supplemental Figure 4B), which expresses an autoactive mutant form of SNC1 protein with enhanced nuclear accumulation (Zhu et al., 2010a; Mang et al., 2012). Again, we observed a nearly complete suppression of the *snc1-1* phenotype as well as dramatically reduced defense gene expression in independent *35S*: *KA120-YFP/snc1-1* transgenic lines (Figure 4A and 4B). These data collectively support a critical role of KA120 in repressing SNC1 activity in plants.

KA120 is capable of altering the nucleocytoplasmic distribution of SNC1 specifically

Because *KA120* encodes a karyopherin-β, we next tested whether KA120 may modulate SNC1 activity by affecting the nucleocytoplasmic distribution of SNC1 or proteins that directly regulate SNC1 activity (e.g., CPR1 that degrades SNC1) or signaling (e.g., EDS1 and PAD4). Consistent with a potential role in regulating nuclear shuttling of cargo, KA120-YFP displays nucleocytoplasmic distribution with concentrated nuclear signal upon transient expression in *Nicotiana benthamiana* and stable expression in *Arabidopsis* (Supplemental Figure 5A and 5B).

To test how KA120 may affect the nucleocytoplasmic distribution of SNC1, we transiently coexpressed SNC1-GFP in *Arabidopsis* protoplasts with KA120-mCherry or free mCherry and quantified the total as well as the nuclear SNC1 signal. We found that SNC1-GFP predominantly localizes to the nucleus upon transient expression; however, compared with free mCherry, coexpression with KA120-mCherry led to a significantly reduced nuclear signal and mildly enhanced cytoplasmic retention of SNC1-GFP (Figure 4C–4E). Nonetheless, the total protein level of SNC1 did not appear to be affected by coexpression with KA120 (Figure 4D [left panel] and Supplemental Figure 5C). The reduced nuclear accumulation of SNC1 in the presence of excessive KA120 was also observed in root cells of *35S*: *SNC1-GFP/35S*: *KA120-mCherry* double transgenic plants (Figure 4F).

In contrast to SNC1, overexpression of KA120 did not significantly affect the nucleocytoplasmic distribution of CPR1, EDS1, and PAD4 in protoplast transient assays (Figure 4E and Supplemental Figure 5F–5H), suggesting that KA120 specifically affects the nucleocytoplasmic distribution of SNC1 protein. Moreover, overexpression of XPO2, an exportin homologous to KA120, was unable to alter the nucleocytoplasmic distribution pattern of SNC1 in protoplast transient assays, and the predominant SNC1 signal remained in the nucleus (Figure 4E; Supplemental Figure 5D and 5E). Together, the above results indicate that KA120 is able to shift the nuclear homeostasis of SNC1 specifically and provide a possible mechanism for its regulation of SNC1 activity.

Reducing SNC1 nuclear retention alleviates the *ka120* phenotype

Lastly, we assessed the potential contribution of excessive nuclear activity of SNC1 to *ka120*-dependent autoimmune activation. We crossed *ka120-1* with *mos7-1* (*modifier of snc1*, *7*), a partial loss-of-function mutation in the NPC component *Nup88* that results in a reduced nuclear level of SNC1 and suppresses the *snc1-1* phenotype (Cheng et al., 2009). We found that the *mos7-1* mutation could partially suppress the stunted growth and defense gene activation in *ka120* plants (Figure 4G and 4H; Supplemental Figure 4C), supporting that SNC1 is overaccumulated or hyperactivated in the nuclei of the *ka120* mutant to contribute to the autoimmune activation. In contrast, a mutation in *importin-α3* (*mos6*) that also partially suppresses the *snc1-1* phenotype has no observable effect on the *ka120* phenotype (Figure 4F and 4G; Supplemental Figure 4C), suggesting that loss of importin-α3 may not significantly influence the nuclear activity of SNC1 in the absence of KA120. These data support a hypothesis that KA120 regulates SNC1 nuclear activity at least partially through coordinating its nucleocytoplasmic distribution.

Karyopherin-β proteins have an ancient origin, and at least 16 subfamilies have been established before the last eukaryotic common ancestor (O'Reilly et al., 2011). They continue to evolve in different eukaryotic species and may have adapted to transport distinct cargoes involved in diverse cellular processes (Tamura and Hara-Nishimura, 2014; Li and Gu, 2020). Among mutant lines of the 18 known *Arabidopsis* karyopherin-β genes, only *ka120* mutants display a *snc1*-like autoimmune phenotype, which can be partially suppressed by compromised SNC1

(G) Partial suppression of *ka120*-induced growth defects by the *mos7-1* but not *mos6* mutant. Three-week-old *Arabidopsis* plants are shown. Scale bar, 1 cm.

(H) Measurement of rosette diameter of 3-week-old WT, *ka120-1*, *ka120-1 mos7-1* (*k/m7*), *ka120-1 mos6* (*k/m6*), *mos7-1*, and *mos6* plants. Data points are shown as blue dots. Tukey's HSD test was performed.

activities. Compared with other karyopherin-β proteins (e.g., XPO2), overexpressing KA120 can specifically alter SNC1 nucleocytoplasmic distribution by significantly reducing SNC1 nuclear signal. However, KA120 overexpression did not appear to alter the nucleocytoplasmic distribution of other major regulators of SNC1 stability or signaling, such as CPR1, EDS1, and PAD4, suggesting a specific role of KA120 overexpression in reducing SNC1 nuclear distribution. At the functional level, overexpressing KA120 could largely suppress SNC1-dependent immune activation in transgenic plants, presumably due to the reduced SNC1 nuclear accumulation or activity. Indeed, reducing the SNC1 nuclear translocation by compromising the NPC alleviates the autoimmune phenotype in *ka120*. These results together support a hypothesis that KA120 functions as a specialized NTR to influence the nuclear transport of SNC1 and emphasize the KA120-dependent pathway as an essential mechanism to gate autoimmune activation in plants. However, whether this process depends on the nuclear transport activity of KA120 needs to be further investigated. Although the physical interaction between KA120 and SNC1 has not been established, there is a possibility that SNC1 is a protein cargo of KA120 for nuclear export. Alternatively, KA120 may mediate nuclear transport of other proteins to affect the nucleocytoplasmic distribution and activity of SNC1. It is also possible that KA120 regulates SNC1 nuclear activity through a nuclear transport-independent mechanism.

It may appear to be a risky evolutionary strategy to prevent aberrant immune activation by relying on a single karyopherin-β to constrain an NLR's nuclear activity; however, one potential benefit is that it may allow differential regulation of nuclear transport pathways. It would be interesting to test whether the activity or transport capacity of KA120 is specifically inhibited during ETI activation as a mechanism to facilitate the nuclear accumulation of SNC1 and enhance immune responses without affecting other karyopherins that mediate immune-unrelated nuclear transport events.

KA120 is a close homolog of human IPO11, which mediates nuclear import of tumor-suppressor PTEN (Chen et al., 2017), a phosphatase that is involved in the regulation of the cell cycle. The *Arabidopsis* PTEN homolog AtPTEN1 has been shown to be essential for pollen development (Gupta et al., 2002). Potential disruption of the nuclear level of AtPTEN1 in the *ka120* mutant may be responsible for the *ka120* sterile phenotype. Notably, none of the identified *ka120* suppressor mutations, including *snc1-r1*, *pad4*, *eds1*, and *mos7*, suppress the sterile phenotype of the *ka120* mutant, supporting that this phenotype is independent of SNC1 activation. Also, these mutations cannot completely rescue the *ka120*-dependent autoimmune activation at both the morphological and transcriptional level, indicating that there might be SNC1/EDS1/PAD4-independent NLRs whose nuclear export is also mediated by KA120, albeit our amiRNA screen was incomplete or not efficient enough to identify them yet. Alternatively, other potential immune regulators that are sufficient to activate immune signaling downstream or in parallel with EDS1/PAD4 may be substrates of KA120. These results suggest that KA120 is a karyopherin that can transport multiple cargoes. Identification of KA120 cargo, including other potential immune regulators, requires the establishment of the KA120 cargo spectrum in the future by newly developed proteomic profiling technology such as proximity labeling proteomics (Xu et al., 2021).

Several well-characterized *Arabidopsis* karyopherin-βs, including HASTY, IMB2, IMB3, and SAD2, have been reported to regulate global microRNA activity and biogenesis (Park et al., 2005; Wang et al., 2011; Cui et al., 2016; Zhang et al., 2017). However, our small RNA-seq analysis for the *ka120* mutant did not reveal significant global changes in microRNAs (Supplemental Figure 6A), and only a limited number of differentially expressed microRNAs were detected compared with WT plants (Supplemental Figure 6B and Supplemental Table 4), suggesting that KA120 may not transport major regulators of microRNA biogenesis and further supporting functional specialization of KA120 among karyopherin-β proteins in plants.

METHODS

Plant material and growth conditions

The *ka120-1* (Salk_148803), *ka120-2* (GABI_605D06), *imb5* (Salk_206109), *plantkap* (Salk_035684), *ipo8-1* (Salk_103333), *sad2* (Salk_133577), and *importin-α3/mos6* (Salk_025919) seeds were obtained from the Arabidopsis Biological Resource Center. The *npr1-2*, *ndr1-1*, *eds1-2*, *eds5-1*, *pad4-1*, and *mos7-1* mutants were obtained from Xinnian Dong's laboratory at Duke University. The *snc1-1* and *snc1-r1* mutants were obtained from Yuelin Zhang's laboratory at the University of British Columbia. The above mutants containing point mutations were crossed with *ka120-1* to generate double mutants. Plants were grown in soil at 22°C under a 12-h-light (110 μE/m^2) and 12-h-dark cycle.

Transgenic plants

The *KA120*, *SNC1*, and *CPR1* genomic DNA (from the start codon until before the stop codon) were cloned into a modified pEarleyGate100 (pEG100) vector with a C-terminal YFP tag using Gateway cloning (Thermo Fisher). The SNC1DN-YFP (Xu et al., 2014) was obtained based on SNC1-YFP using a Q5 Site-Directed Mutagenesis Kit (NEB). The artificial microRNA targeting *KA120* was cloned into pBAV154 containing a dex-inducible promoter. All primers used for cloning are listed in Supplemental Table 5. The *KA120-YFP*, *SNC1DN-YFP*, and *CPR1-YFP* constructs were transformed into the *ka120-1* heterozygous background. T1 transgenic lines were screened in soil supplemented with Basta, and the expression of transgenes was confirmed by fluorescence microscopy. T2 plants were genotyped using PCR to obtain *ka120-1* homozygous plants. The *KA120-YFP* construct was also transformed into the *snc1-1* background. The dex-inducible *KA120*-amiRNA construct was transformed into Col-0 background and T3 transgenic plants were used for experiments. For overexpression of SNC1, a translational fusion of *SNC1* genomic DNA with GFP was cloned into pCambia1300, and the construct was transformed into the WT background. All transgenic lines were generated by the agrobacteria-mediated floral-dipping method.

Artificial microRNA library-based *ka120* suppressor screen

Vectors expressing amiRNA were constructed as described previously (Schwab et al., 2005). In brief, we designed 116 amiRNA (21mer) that target 108 *Arabidopsis* NLR genes (Lolle et al., 2017) using the WDR3 tool (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). The step-loop structured fragments were generated by overlapping PCR, and the product was inserted into Gateway entry vector pDONR207 using BP cloning and subsequently cloned into destination vector pEG100 using LR cloning (Thermo Fisher). All primers used for cloning amiRNA constructs are listed in Supplemental Table 2. An equal amount of these 116 amiRNA destination constructs were aliquoted and mixed to obtain the construct library, which was then bulk transformed into *Agrobacterium* strain GV3101. Bacterial transformants were bulk collected and cultured for

plant transformation into *ka120-1* heterozygous plants. T1 plants were screened by Basta in soil and genotyped to identify *ka120-1* homozygous individuals. Genomic DNA from lines with suppression phenotype was extracted, and the amiRNA construct was amplified and sequenced to identify potential NLR targets.

RNA extraction and qRT–PCR

Total RNA was extracted from plant tissues using TRIzol RNA reagent (Sigma). First-strand cDNA was synthesized from 1 μg of total RNA using the Maxima H Minus reverse transcriptase (Thermo Fisher). qPCR was performed using SYBR Select Master Mix (Thermo Fisher). Primers used for qPCR are provided in Supplemental Table 5. The expression of *UBQ5* and *ACT2* were used as reference genes.

Microarray and RNA-seq experiments

For microarray experiments, the entire rosette of 3-week-old WT and *ka120-1* plants were collected for total RNA preparation using an RNAprep pure Plant Kit (TIANGEN Biotech) before being subjected to Affymetrix *Arabidopsis* ATH1 Genome Array (Agilent). The resulting dataset was deposited in the Gene Expression Omnibus (GEO: GSE147683). For RNA-seq analysis, total RNA was isolated from the entire rosette of 3-week-old WT, *ka120-1*, *ka120-1 pad4-1*, and *ka120-1 ndr1-1* plants. cDNA generation, mRNA-seq library construction, quality control, library sequencing using Illumina Novaseq 6000, mapping of sequence reads to *Arabidopsis* reference genome (TAIR10), and generation of the total number of uniquely mapped reads was conducted by Novogene Bioinformatic Technology. The resulting dataset was deposited in the Gene Expression Omnibus (GEO: GSE147682). Three biological replicates were included in both microarray and RNA-seq experiments. DEGs were analyzed using GeneSpring 13.0 (for microarray) and DESeq2 package in R (for RNA-seq). False discovery rate <0.05 and fold change >2 were set as cutoffs for significant DEGs. The factoextra package in R was used for hierarchical clustering, and the pheatmap package in R was used for generating heatmaps. GO enrichment analysis was conducted using agriGO (v2.0) (http://systemsbiology.cau.edu.cn/agriGOv2/index.php). For the small RNA-seq experiment, rosette leaves of 3-week-old WT and *ka120-1* mutant plants were collected and sent to Novogene for RNA extraction. Sequencing libraries were generated using NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB) and were sequenced on an Illumina HiSeq 2500/2000 platform. Raw data were processed via custom perl and Python scripts, and small RNA reads were calculated and normalized in transcript per million mapped reads. Differentially expressed small RNAs were analyzed using the DESeq2 package in R using p value <0.05 and $\log_2$(fold change) >1 as cutoffs.

Protoplast transfection and microscopy imaging

The genomic DNA (from the start codon until before the stop codon) of *KA120* and *XP O 2* were cloned into pUC18-mCherry by In-Fusion cloning (Vazyme). The *SNC1* genomic DNA was cloned into pTF486-GFP using restriction enzymes *Sal*I and *Bam*HI. All constructions were purified using a ZymoPURE II Plasmid Maxiprep kit (Zymo Research). *Arabidopsis* mesophyll protoplasts were prepared and transfected as previously described (Yoo et al., 2007). GFP and mCherry signals were detected using a Zeiss AxioImager M1 fluorescence microscope equipped with a 40× oil objective lens. The indicated numbers of randomly selected protoplasts with mCherry and GFP expression were quantified for fluorescence signal intensity in the nucleus and the whole cell using Fiji.

DATA AVAILABILITY

All RNA-seq and microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO: GSE147682 and GSE147683).

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Molecular Plant Online*.

FUNDING

X. Shen and X. Shi were supported by Tsinghua-Peking Joint Center for Life Sciences. This project was supported by the USDA National Institute of Food and Agriculture (HATCH project CA-B-PLB-0243-H), the National Science Foundation (grant MCB-2049931), and startup funds from Innovative Genomics Institute and University of California Berkeley.

AUTHOR CONTRIBUTIONS

M.J., X. Shen, and Y.G. designed the research. M.J., X. Shen, Y.T., and X. Shi performed the experiments. M.J. and Y.G. wrote the paper.

ACKNOWLEDGMENTS

We thank Drs. Shauna Somerville and Chao Wang for help with the powdery mildew assay. No conflict of interest declared.

Received: May 17, 2021
Revised: June 15, 2021
Accepted: June 17, 2021
Published: June 17, 2021

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