

# Activation of Plant Immune Responses by a Gain-of-Function Mutation in an Atypical Receptor-Like Kinase<sup>1[C][W][OA]</sup>

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*Arabidopsis* (*Arabidopsis thaliana*) suppressor of *npr1-1*, *constitutive1* (*snc1*) contains a gain-of-function mutation in a Toll/interleukin receptor-nucleotide binding site-leucine-rich repeat Resistance (R) protein and it has been a useful tool for dissecting R-protein-mediated immunity. Here we report the identification and characterization of *snc4-1D*, a semidominant mutant with *snc1*-like phenotypes. *snc4-1D* constitutively expresses defense marker genes *PR1*, *PR2*, and *PDF1.2*, and displays enhanced pathogen resistance. Map-based cloning of *SNC4* revealed that it encodes an atypical receptor-like kinase with two predicted extracellular glycerophosphoryl diester phosphodiesterase domains. The *snc4-1D* mutation changes an alanine to threonine in the predicted cytoplasmic kinase domain. Wild-type plants transformed with the mutant *snc4-1D* gene displayed similar phenotypes as *snc4-1D*, suggesting that the mutation is a gain-of-function mutation. Epistasis analysis showed that NON-RACE-SPECIFIC DISEASE RESISTANCE1 is required for the *snc4-1D* mutant phenotypes. In addition, the *snc4-1D* mutant phenotypes are partially suppressed by knocking out *MAP KINASE SUBSTRATE1*, a positive defense regulator associated with *MAP KINASE4*. Furthermore, both the morphology and constitutive pathogen resistance of *snc4-1D* are partially suppressed by blocking jasmonic acid synthesis, suggesting that jasmonic acid plays an important role in *snc4-1D*-mediated resistance. Identification of *snc4-1D* provides us a unique genetic system for analyzing the signal transduction pathways downstream of receptor-like kinases.

Receptor-like kinases (RLKs) are a large group of kinases with a variable extracellular domain and a cytoplasmic kinase domain linked by a single transmembrane motif. RLKs have been shown to play diverse roles in regulating plant innate immunity as well as growth and development (Morillo and Tax, 2006). The extracellular domains of RLKs are believed to bind directly to ligands to perceive extracellular signals, whereas the cytoplasmic kinase domains transduce these signals into the cell. There are over 600 RLKs (Shiu and Bleecker, 2001) in *Arabidopsis* (*Arabidopsis thaliana*). The biological functions of most RLKs are unknown.

Several RLKs have been identified to be receptors of microbe-associated molecular patterns (MAMPs). *FLS2*

and *EFR* are two well-characterized RLKs with extracellular Leu-rich repeats (LRRs) that recognize bacterial flagellin and translation elongation factor EF-Tu, respectively (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). *BAK1* is also an RLK with extracellular LRRs. *BAK1* seems to function as an adaptor protein for multiple RLKs including *BRI1*, *FLS2*, and *BIR1* (Li et al., 2002; Nam and Li, 2002; Chinchilla et al., 2007; Heese et al., 2007; Gao et al., 2009). Interestingly, knocking out *BIR1* activates cell death and defense responses mediated by another RLK, *SOBIR1* (Gao et al., 2009). Recently, the rice (*Oryza sativa*) RLK *Xa21* was also suggested to be a MAMP receptor. *Xa21* recognizes a peptide derived from the secreted effector protein *AvrXa21*, which is conserved among different *Xanthomonas* species (Lee et al., 2009). Unlike *FLS2*, *EFR*, and *Xa21*, the putative receptor for chitin is an RLK with three extracellular LysM domains instead of LRRs that are required for the perception of chitin as well as resistance against bacterial pathogens (Miya et al., 2007; Wan et al., 2008; Gimenez-Ibanez et al., 2009).

Perception of MAMPs by receptors leads to the rapid activation of mitogen-activated protein (MAP) kinases including *MAP KINASE3* (*MPK3*), *MPK4*, and *MPK6* (Boller and Felix, 2009). *MAP KINASE SUBSTRATE1* (*MKS1*) was identified as an *MPK4*-interacting protein that positively regulates defense responses (Andreasson et al., 2005). Silencing of *MKS1*

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compromises basal resistance to *Pseudomonas syringae* pv *tomato* DC3000, whereas overexpression of *MKS1* leads to enhanced pathogen resistance. Activation of MAMP receptors also induces a number of responses such as oxidative burst, callose deposition, and increased salicylic acid synthesis (Boller and Felix, 2009). Defense responses induced by different MAMPs seem similar, suggesting that they may share common signaling components. Identification of the signaling components downstream of RLK receptors remains a major task in understanding MAMP-triggered immunity.

Here we report the identification and characterization of *suppressor of npr1-1, constitutive4-1D (snc4-1D)*, a gain-of-function mutant of an atypical RLK that is autoactivated by a mutation in its kinase domain. The *snc4-1D* mutant plants constitutively express defense marker genes *PR1*, *PR2*, and *PDF1.2*, and display enhanced resistance to *Hyaloperonospora arabidopsidis* (*H. a.*) Noco2. Epistasis analysis showed that *snc4-1D*-mediated defense responses are dependent on multiple factors including NON-RACE-SPECIFIC DISEASE RESISTANCE1 (*NDR1*), *MKS1*, and *OPR3*.

## RESULTS

### Identification and Characterization of *snc4-1D*

Our lab has been interested in identifying novel negative regulators of plant immunity (Gao et al., 2008). Loss-of-function mutations in these negative regulators would yield mutants with enhanced pathogen resistance and dwarfism that is usually associated with strong resistance phenotypes. Very rarely, gain-of-function mutations in positive regulators in the same pathways would yield mutants with similar phenotypes, such as in *snc1* (Li et al., 2001). When we screened an ethyl methanesulfonate (EMS)-mutagenized population in the Columbia-0 (*Col-0*) background for mutants with enhanced resistance against the oomycete pathogen *H. a.* Noco2, one of the mutants, designated as *snc4-1D*, exhibited *snc1*-like dwarfism (Fig. 1A) and strong resistance to the virulent pathogen *H. a.* Noco2 (Fig. 1B). Plants heterozygous for *snc4-1D* have intermediate sizes, suggesting the mutant is semidominant and the mutation is most likely a gain-of-function mutation in a positive regulator of defense. Real-time reverse transcription (RT)-PCR analysis showed *snc4-1D* constitutively expresses defense marker genes *PR1* (Fig. 1C), *PR2* (Fig. 1D), and *PDF1.2* (Fig. 1E). These data suggest that defense responses are constitutively activated in *snc4-1D*.

Mutants with enhanced disease resistance often accumulate high levels of hydrogen peroxide ( $H_2O_2$ ) and exhibit spontaneous cell death. To test whether *snc4-1D* accumulates  $H_2O_2$ , 3, 3'-diaminobenzidine (DAB) staining was performed on the *snc4-1D* mutant seedlings. Strong staining was observed in *snc4-1D* mutant seedlings (Fig. 1F), indicating that *snc4-1D* accumulated high levels of  $H_2O_2$  compared to wild type.

In addition, microscopic cell death was also observed in the *snc4-1D* mutant from trypan blue staining (Fig. 1G).

### Map-Based Cloning of *snc4-1D*

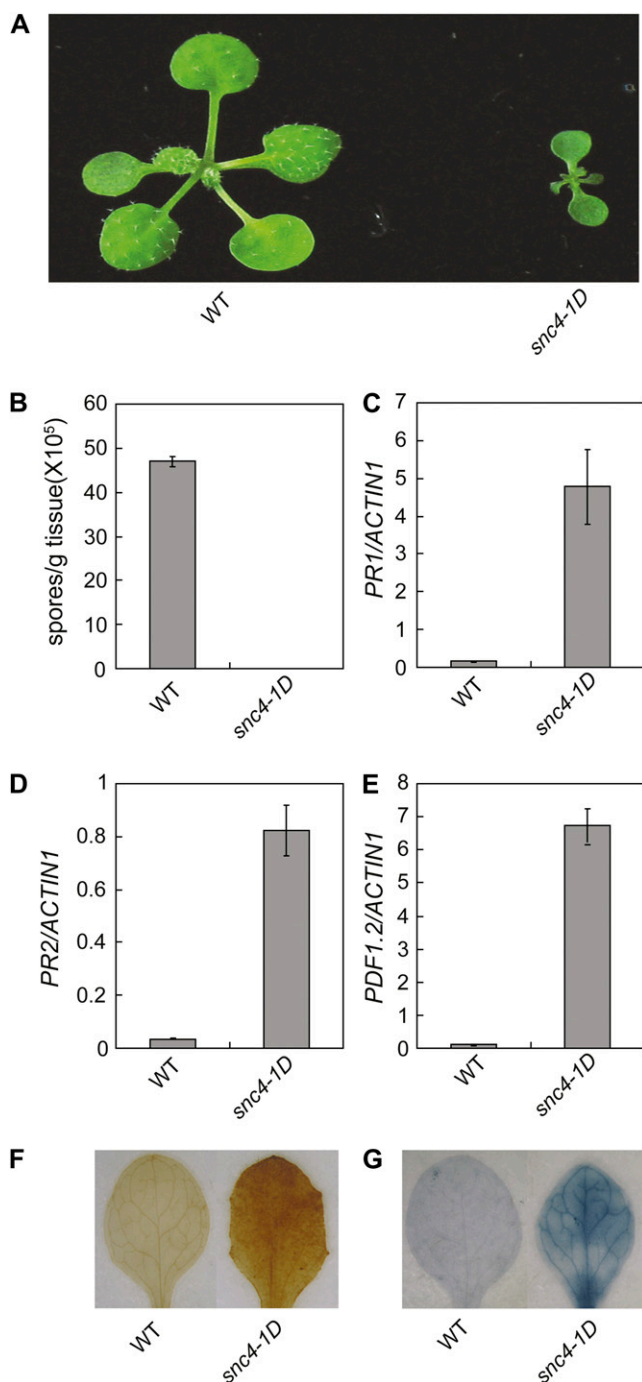
To map the *snc4-1D* mutation, *snc4-1D* was crossed with *Landsberg erecta* (*Ler*). Plants with *snc4-1D* morphology were selected from the F2 progeny and used for crude mapping. The mutation was initially mapped to a region between F13O11 and F20P5 on chromosome 1 (Fig. 2A). Further analysis of approximately 1,100 F2 plants flanked the mutation to a 35-kb region between marker T4O24 and F1O19. The coding sequence of genes in the region were amplified by PCR from the genomic DNA of *snc4-1D* and sequenced. A single G to A mutation was found in one of the sequenced genes, *At1g66980* (Fig. 2B). Previously it was reported that a knockout mutant of *At1g66980* displayed wild-type morphology (Hayashi et al., 2008), suggesting that the mutant phenotypes observed in *snc4-1D* is not likely caused by loss of function of the protein encoded by *At1g66980*.

*At1g66980* encodes a predicted RLK (Fig. 2C). The extracellular region of this protein contains two putative glycerophosphoryl diester phosphodiesterase (GDPD) domains. The protein also has a predicted N-terminal signal peptide and a single transmembrane domain between the cytoplasmic kinase domain and the extracellular GDPD domains. Originally identified in *Escherichia coli*, GDPD catalyzes the hydrolysis of a glycerophosphodiester to an alcohol and glycerol 3-P (Larson et al., 1983). GDPD activity was also detected in cell wall fractions of carrot (*Daucus carota*; Van Der Rest et al., 2004). Blast analysis failed to identify any other RLK with extracellular GDPD domains in Arabidopsis and other plants, suggesting that *At1g66980* probably evolved recently through combining its GDPD and kinase domains. To confirm that the GDPD and kinase domains are encoded in the same gene, the size of the *SNC4* cDNA is determined by RT-PCR (Supplemental Fig. S1A). In addition, western-blot analysis showed that the *SNC4*-GFP fusion protein expressed in transgenic plants has a mass of about 150 kD, close to the predicted size of the fusion protein (Supplemental Fig. S1B).

The *SNC4*-GFP fusion protein localized to the plasma membrane (Supplemental Fig. S2), suggesting that *SNC4* is a plasma membrane protein as predicted. The mutation in *snc4-1D* changes the Ala 850 to Thr in the kinase domain of the protein, which does not change the localization of the protein (Supplemental Fig. S2).

### Complementation

Since T-DNA knockout mutants of *At1g66980* did not display *snc4-1D* phenotypes, we deduced that the Ala-850 to Thr mutation is most likely a gain-of-function mutation in *At1g66980*. If that is the case, transforming the mutant version of *At1g66980* in wild-



**Figure 1.** Characterization of the *snc4-1D* mutant. A, Morphology of wild type (WT) and *snc4-1D*. Plants were grown on soil and photographed 3 weeks after planting. B, Growth of *H. a. Noco2* on wild-type and *snc4-1D* mutant plants. Two-week-old seedlings were sprayed with *H. a. Noco2* spores ( $5 \times 10^4$  spores/mL). Infection was scored 7 d later by counting the number of conidia spores with a hemocytometer. The values presented are averages of three replicates  $\pm$  SDs. C to E, Expression of *PR1* (C), *PR2* (D), and *PDF1.2* (E) in wild-type and *snc4-1D* mutant seedlings. Total RNA was extracted from 2-week-old seedlings grown on one-half-strength Murashige and Skoog medium at 23°C. Relative levels of *PR1*, *PR2*, and *PDF1.2* were determined by real-time PCR. Values were normalized to the expression of *ACTIN1*. Error bars represent SD from three measurements. F, DAB staining of true

type background would yield plants with *snc4-1D* phenotypes. When we transformed constructs expressing the wild-type or mutant protein of At1g66980 under the control of its native promoter into wild-type plants, all transgenic lines obtained for the wild-type *SNC4* construct displayed wild-type morphology. In contrast, about half of the transgenic lines with the mutant *snc4-1D* construct exhibit *snc4-1D*-like morphology (Fig. 3A). Real-time PCR analysis of defense marker genes *PR1*, *PR2*, and *PDF1.2* showed that these marker genes were constitutively expressed in the transgenic plants carrying the mutant *snc4-1D* gene but not in the transgenic plants expressing the wild-type *SNC4* gene (Fig. 3, B–D). In addition, transgenic plants expressing the mutant At1g66980 displayed enhanced resistance to *H. a. Noco2* (Fig. 3E). Progeny from a representative line expressing the wild-type or mutant gene were further analyzed for the accumulation of H<sub>2</sub>O<sub>2</sub> and cell death. As shown in Supplemental Figure S3, strong DAB and trypan blue staining was observed in the transgenic line carrying the mutant *snc4-1D* transgene but not in the line with the wild-type *SNC4* transgene. Thus, all the *snc4-1D* mutant phenotypes can be recaptured by expressing the At1g66980 mutant gene in wild-type plants, indicating that *SNC4* is At1g66980 and the mutation in *snc4-1D* is a gain-of-function mutation that activates downstream defense responses.

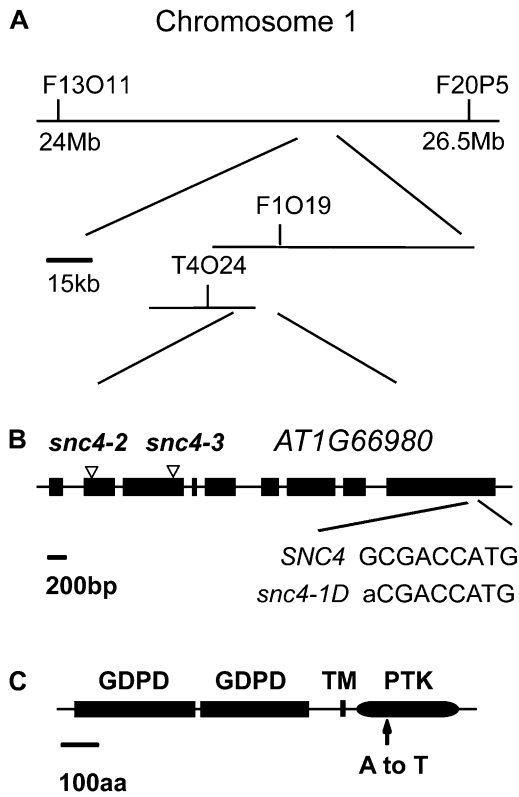
#### Analysis of Intragenic Suppressors of *snc4-1D*

To identify mutants that suppress the *snc4-1D* mutant phenotypes, we mutagenized *snc4-1D* seeds with EMS and screened for mutants with wild-type morphology in the M2 population. Twenty-one *snc4-1D* suppressor mutants were obtained from the screen. Because *snc4-1D* seems to be a gain-of-function mutation, we hypothesized that some of the mutants recovered may be intragenic suppressors of *snc4-1D*. Sequence analysis of the *SNC4* locus identified six mutants containing mutations in *snc4-1D* (Fig. 4A). Among these mutations, one is a missense mutation in one of the GDPD domains, two are missense mutations in the kinase domain, and the rest are nonsense mutations that cause early truncations of the protein (Fig. 4B). Real-time RT-PCR analysis showed that suppression of the *snc4-1D* mutant morphology correlates with reduced expression of *PR1*, *PR2*, and *PDF1.2* (Fig. 4, C–E). These data further confirm that At1g66980 is *SNC4* and *snc4-1D* is a gain-of-function mutation.

#### NDR1 Is Required for the Mutant Phenotypes of *snc4-1D*

Arabidopsis NDR1 is generally required for resistance mediated by coiled coil-nucleotide binding site (NB)-LRR Resistance (R) proteins, whereas EDS1 is required for resistance mediated by many Toll/inter-

leaves of wild-type and mutant plants. G, Trypan blue staining of true leaves of wild-type and mutant plants.



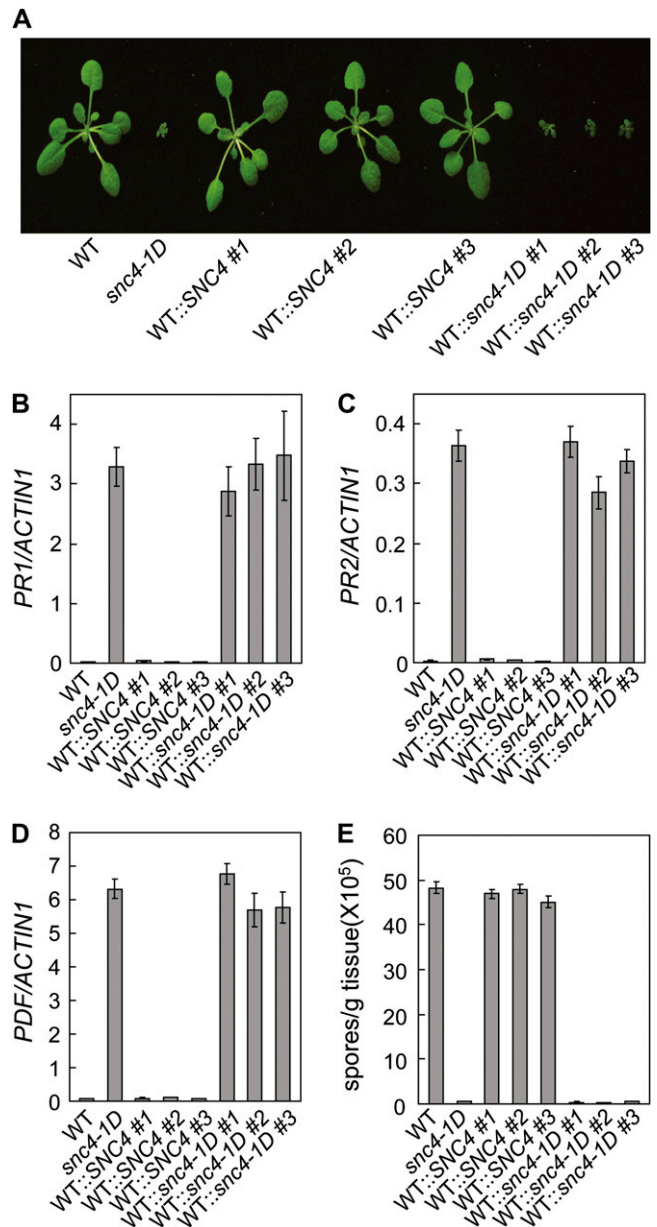
**Figure 2.** Map-based cloning of *snc4-1D*. A, Map of the *snc4-1* locus. B, Exon/intron structure of *SNC4*. The coding regions are indicated with boxes. *snc4-2*, Salk\_122292; *snc4-3*, WiscDsLox444C5. C, Predicted protein structure of *SNC4*. TM, Transmembrane motif; PTK, protein Tyr kinase. The locations of the mutations in *SNC4* are as indicated. aa, Amino acids.

leukin receptor-NB-LRR R proteins (Century et al., 1995; Parker et al., 1996; Aarts et al., 1998). To test whether *snc4-1D* activates NDR1 or EDS1-dependent signaling pathways, *snc4-1D* was crossed with *ndr1-1* or *eds1-2* (backcrossed into Col background). As shown in Figure 5A, the *snc4-1D* mutant morphology was largely suppressed by *ndr1-1* but not by *eds1-2*. Analysis of *PR1* and *PR2* expression in the double mutants showed that the constitutive expression of both *PR1* and *PR2* in *snc4-1D* was largely blocked by *ndr1-1*. In contrast, *PR1* and *PR2* expression were only partially affected by the *eds1-2* mutation (Fig. 5, B and C). The elevated *PDF1.2* expression in *snc4-1D* is partially suppressed by *ndr1-1* and to a lesser extent by *eds1-2* (Fig. 5D). We further tested whether resistance to *H. a. Noco2* in *snc4-1D* is affected in the double mutants. As shown in Figure 5E, resistance to *H. a. Noco2* was reduced in both *snc4-1D ndr1-1* and *snc4-1D eds1-2*.

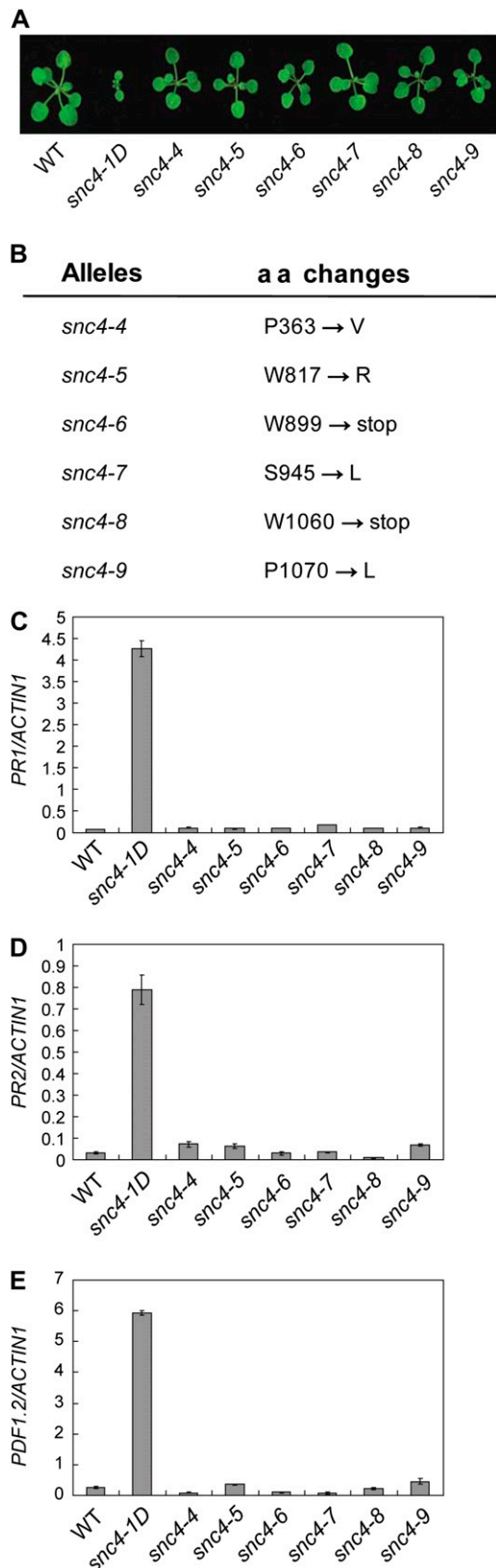
**MKS1 Is Required for *snc4-1D*-Mediated Resistance Responses**

RLKs that serve as MAMP receptors, such as FLS2 and EFR, signal through MAP kinase cascades (Boller

and Felix, 2009). If *snc4-1D* signals through a similar cascade, one would expect that mutation in a positive regulator of the MAP kinase pathway would block *snc4-1D*-mediated immunity. Arabidopsis MKS1 is a positive regulator of defense responses that interacts



**Figure 3.** Complementation analysis of *snc4-1D*. A, Morphology of wild-type (WT), *snc4-1D*, WT::*SNC4* (wild type transformed with a *SNC4* genomic clone), and WT::*snc4-1D* (wild type transformed with a *snc4-1D* genomic clone) transgenic lines. All plants were grown on soil and photographed when they were 3 weeks old. B to D, Expression of *PR1* (B), *PR2* (C), and *PDF1.2* (D) in wild-type, *snc4-1D*, WT::*SNC4*, and WT::*snc4-1D* transgenic lines. Values were normalized to the expression of *ACTIN1*. Error bars represent sd from averages of three measurements. E, Growth of *H. a. Noco2* on wild-type, *snc4-1D*, WT::*SNC4*, and WT::*snc4-1D* transgenic lines. Infection was performed and scored as in Figure 1B. The values presented are averages of three replicates  $\pm$  sd. [See online article for color version of this figure.]



**Figure 4.** Characterization of the intragenic suppressors of *snc4-1D*. **A**, Morphology of wild-type (WT), *snc4-1D*, and the intragenic suppressor mutant plants. Plants were grown on soil at 23°C and photographed 3

with MPK4 (Andreasson et al., 2005). When we made the *snc4-1D mks1-1* double mutant, the morphological phenotypes of *snc4-1D* were largely suppressed by *mks1-1* (Fig. 6A). Real-time RT-PCR analysis showed that the elevated expression of *PR1* in the *snc4-1D* single mutant was blocked in the *snc4-1D mks1-1* double mutant plants (Fig. 6B). In contrast, the expression of *PR2* and *PDF1.2* expression in *snc4-1D* is only modestly affected by *mks1-1* (Fig. 6, C and D). Analysis of resistance to *H. a. Noco2* showed that the enhanced resistance observed in *snc4-1D* was largely suppressed by *mks1-1* (Fig. 6E).

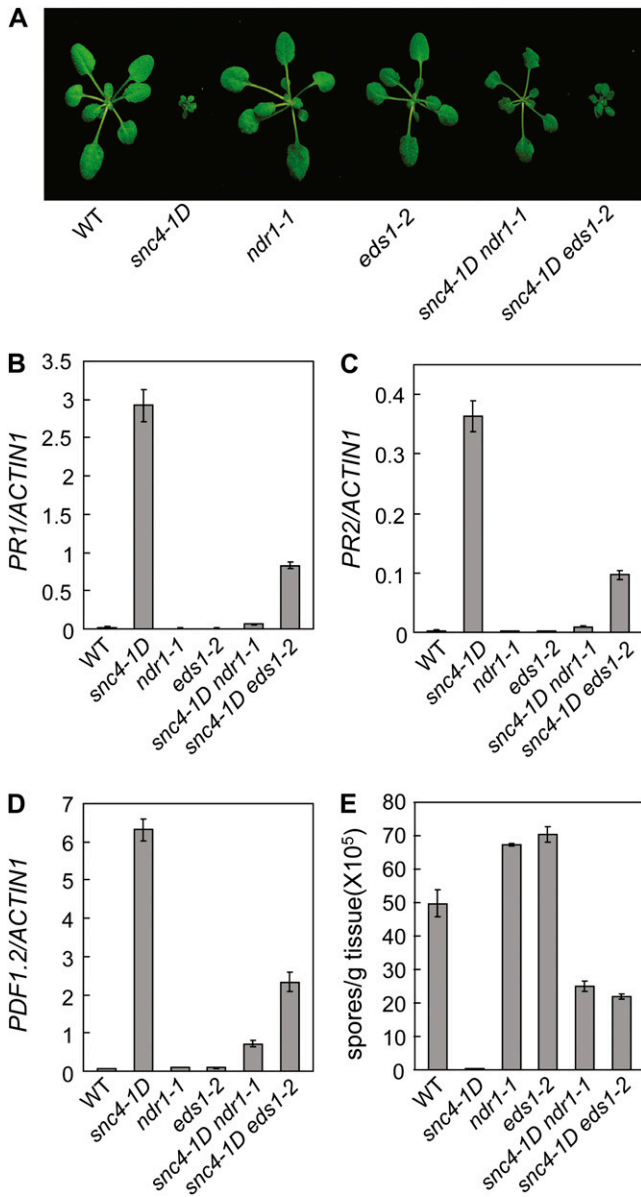
#### JA Is Required for *snc4-1D*-Mediated Resistance Responses

Since *snc4-1D* constitutively expresses *PDF1.2*, a defense marker gene of the jasmonic acid (JA) pathway, we tested whether JA is required for the *snc4-1D* mutant phenotypes by crossing *snc4-1D* into *opr3-2*, a loss-of-function mutant of 12-oxo-phytodienoic acid reductase 3 that is required for jasmonate biosynthesis (Stintzi and Browse, 2000). As shown in Figure 7A, the *snc4-1D opr3-2* double mutant has intermediate size compared to the wild-type and *snc4-1D* mutant plants, suggesting that *opr3-2* partially suppresses the *snc4-1D* mutant phenotype. *PDF1.2* expression in *snc4-1D* was dramatically lowered by *opr3-2* (Fig. 7B). Surprisingly, the expression of *PR1* (Fig. 7C) and *PR2* (Fig. 7D) in *snc4-1D* is also largely suppressed by *opr3-2*. When *snc4-1D opr3-2* plants were challenged by *H. a. Noco2*, the pathogen grew much better on the double mutant than on *snc4-1D*, but at a lower level than on wild-type plants and *opr3-2*, suggesting that JA is required for pathogen resistance activated by *snc4-1D* (Fig. 7E).

#### DISCUSSION

Previous studies have identified several gain-of-function mutations in NB-LRR R genes that lead to autoactivation of defense responses (Shirano et al., 2002; Zhang et al., 2003a; Noutoshi et al., 2005; Igari et al., 2008). One of the mutants, *snc1*, has been used to identify components involved in R-protein activation (Palma et al., 2005, 2007; Zhang et al., 2005; Zhang and Li, 2005; Goritschnig et al., 2007, 2008; Cheng et al., 2009). The *snc4-1D* mutation reported here results in activation of an atypical RLK with an unusual extracellular region consisting of two GDPD domains. SNC4 appears to be a unique RLK with no clear orthologs in other plant species. Its kinase domain is

weeks after planting. **B**, Mutations identified in the intragenic suppressor mutant alleles. **C** to **E**, Expression of *PR1* (**C**), *PR2* (**D**), and *PDF1.2* (**E**) in wild type, *snc4-1D*, and the suppressor mutants of *snc4-1D*. Values were normalized to the expression of *ACTIN1*. Error bars represent SD from averages of three measurements. aa, Amino acid. [See online article for color version of this figure.]

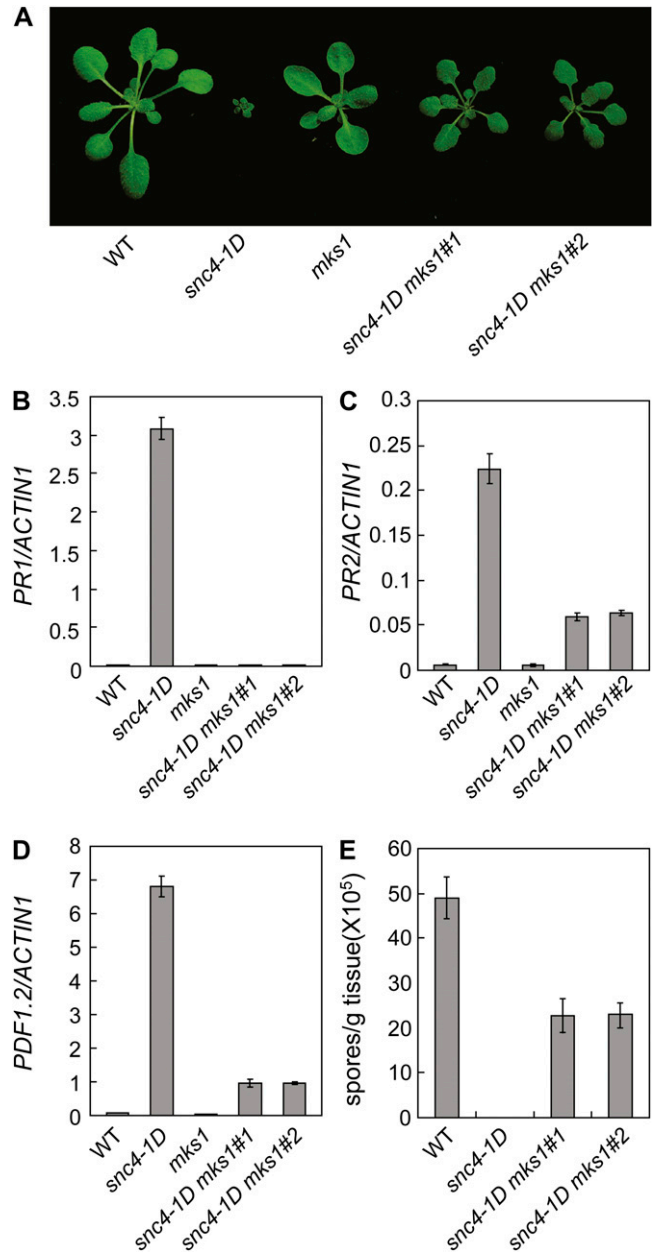


**Figure 5.** Analysis of the *snc4-1D ndr1-1* and *snc4-1D eds1-2* double mutants. A, Morphology of 3-week-old plants of the indicated genotypes. B to D, Expression of *PR1* (B), *PR2* (C), and *PDF1.2* (D) in the indicated genotypes. Values were normalized to the expression of *ACTIN1*. Error bars represent  $\pm$  SD from averages of three measurements. E, Growth of *H. a. Noco2* on the indicated genotypes. Infection was performed and scored as in Figure 1B. The values presented are averages of three replicates  $\pm$  SD. [See online article for color version of this figure.]

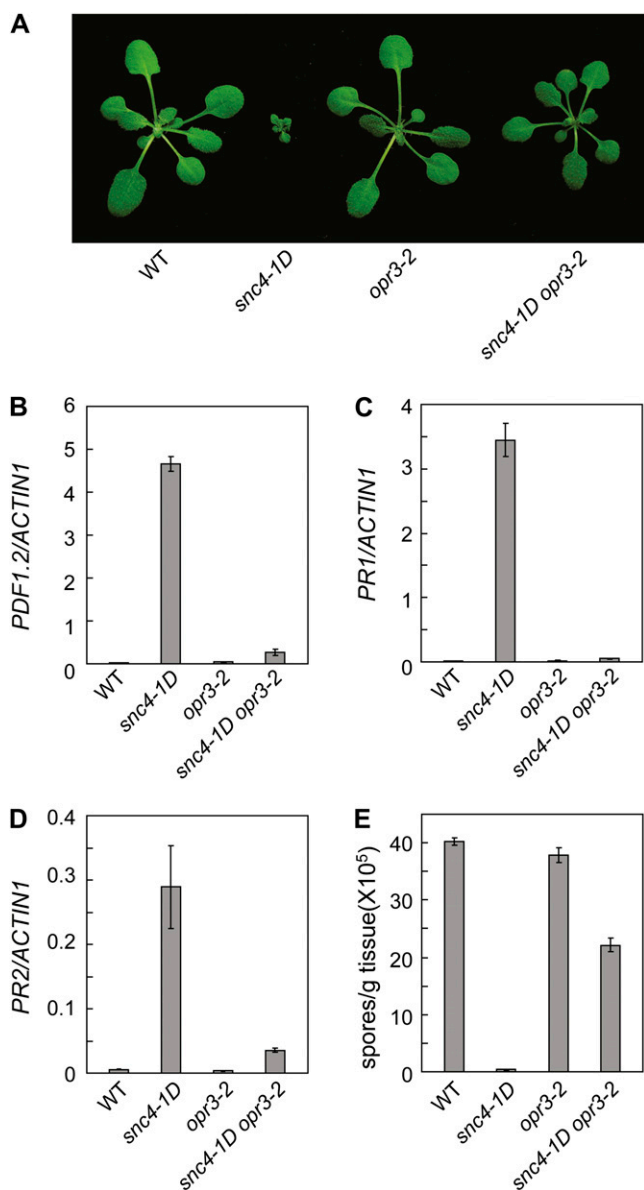
most similar to RLKs in the LRK10L-2 subfamily (Shiu and Bleecker, 2001). Functions of RLKs in this subfamily are unknown. The mutation in *snc4-1D* is located in an unconserved region within subdomain III of the kinase. No similar gain-of-function mutations in other RLKs have been previously identified to constitutively activate downstream signaling pathways. The mechanism on how the mutation in

*snc4-1D* activates defense responses remains to be determined.

In *Arabidopsis*, there are six GPPD domain-containing proteins that are closely related to the extracellular domain of *SNC4* (Supplemental Fig. S4).



**Figure 6.** Analysis of *snc4-1D mks1-1* double mutant. A, Morphology of 3-week-old wild type (WT), *mks1-1*, *snc4-1D*, and two representative lines for the *snc4-1D mks1-1* double mutant. B to D, Expression of *PR1* (B), *PR2* (C), and *PDF1.2* (D) in wild-type, *mks1-1*, *snc4-1D*, and *snc4-1D mks1-1* seedlings. Values were normalized to the expression of *ACTIN1*. The values presented are averages of three replicates  $\pm$  SD. E, Growth of *H. a. Noco2* on wild-type, *snc4-1D*, and *snc4-1D mks1-1* plants. Infection was performed and scored as in Figure 1B. The values presented are averages of three replicates  $\pm$  SD. [See online article for color version of this figure.]



**Figure 7.** Characterization of *snc4-1D opr3-2* double mutants. A, Morphology of wild-type (WT), *snc4-1D*, *opr3-2*, and *snc4-1D opr3-2* mutant plants. Plants were grown on soil and photographed when they were 3 weeks old. B to D, Expression of *PDF1.2* (B), *PR1* (C), and *PR2* (D) in wild-type, *snc4-1D*, *opr3-2*, and *snc4-1D opr3-2* mutant seedlings. Error bars represent  $\pm$  SD from averages of three measurements. Values were normalized to the expression of *ACTIN1*. E, Growth of *H. a. Noco2* on wild-type, *snc4-1D*, *opr3-2*, and *snc4-1D opr3-2* double mutant plants. Infection was performed and scored as in Figure 1B. The values presented are averages of three replicates  $\pm$  SD. [See online article for color version of this figure.]

One of the proteins, SHV3, is involved in the cell wall organization (Hayashi et al., 2008). Sequence analysis showed that some key residues involved in binding to substrates and calcium ions in the *E. coli* GDPD are not conserved in SHV3 and related proteins (Hayashi et al., 2008). These residues are not conserved in

SNC4 either. It suggests that SHV3, SNC4, and related proteins may not have the same enzymatic activity as the GDPD in *E. coli*.

The GDPD domain of SNC4 could be involved in perception of lipid-derived molecules from microbial pathogens or damage responses. Interestingly, one of the intragenic suppressors of *snc4-1D* (*snc4-4*) contains a mutation in the GDPD domain. Western-blot analysis showed that this mutation does not affect the stability of the protein (Supplemental Fig. S5), suggesting that the GDPD domain may be important for the function of SNC4. It is unclear how the *snc4-4* mutation affects the function of the protein. Additional mutagenesis analysis of the GDPD domain of SNC4 will help us better understand its function.

To analyze downstream signaling pathways activated in *snc4-1D*, we carried out epistasis analysis between *snc4-1D* and several known mutants involved in pathogen resistance. One unexpected finding is that the mutant morphology as well as activation of defense gene expression and pathogen resistance in *snc4-1D* is suppressed by *ndr1-1*, a mutant previously known to be defective in resistance mediated by a number of coiled coil-NB-LRR R proteins (Century et al., 1995). In contrast, the *eds1-2* mutation does not affect the mutant morphology and *PR2* expression in *snc4-1D*. However, resistance to *H. a. Noco2* and expression of *PR1* are reduced in the *snc4-1D eds1-2* double mutant. Since EDS1 is also required for basal resistance (Parker et al., 1996), reduced resistance to *H. a. Noco2* could be caused by lowered basal resistance by *eds1-2*.

Another mutation that partially suppresses the *snc4-1D* mutant phenotypes is *mks1*. MKS1 has previously been shown to be a positive regulator of defense responses, where it is phosphorylated in response to bacterial pathogen infections (Andreasson et al., 2005). Our results suggest that MKS1 functions downstream of the RLK SNC4, suggesting that MAP kinase signaling could participate in *snc4-1D*-mediated immunity. It will be interesting to determine whether *snc4-1D* activates MKS1 through a MAP kinase cascade that functions downstream of the known MAMP receptors.

Epistasis analysis showed that JA-dependent resistance pathways are activated in *snc4-1D*. Blocking JA synthesis suppresses the morphological phenotypes as well as constitutive expression of *PDF1.2*, *PR1*, and *PR2* in *snc4-1D*. While it is known that expression of *PDF1.2* is regulated by JA, activation of *PR1* and *PR2* are often associated with salicylic acid-induced defense response. How JA modulates the expression of *PR1* and *PR2* in *snc4-1D* remains to be determined.

Based on our data, a working model is proposed (Supplemental Fig. S6). SNC4 probably encodes a pathogen-associated molecular pattern or damage-associated molecular pattern receptor candidate. Perception of the pathogen-associated molecular pattern or damage-associated molecular pattern signal leads to activation of SNC4 and downstream defense pathways. Since mutations in either *NDR1* or *OPR3* par-

tially suppress the mutant phenotypes of *snc4-1D* and these mutations have different effects on the expression of defense marker genes, NDR1 and OPR3 may function in parallel downstream of SNC4. The stronger effect of *ndr1-1* on *PR1* and *PR2* expression than *mks1-1* suggests that NDR1 may function in earlier events of the signaling pathways comparing to MKS1. We tentatively placed NDR1 upstream of the MAP kinase cascade. Additional studies are required to identify the upstream signal of SNC4 and to better define the relationships between NDR1, OPR3, MKS1, and MAP kinase cascades activated in *snc4-1D*.

In conclusion, we have identified a unique gain-of-function mutant of an atypical RLK. Epistasis analysis showed that *snc4-1D* activates multiple downstream resistance pathways. Our results suggest that *snc4-1D* could be utilized as a powerful tool for genetic analysis of resistance pathways activated by RLKs. Further characterization and cloning of the *snc4-1D* extragenic suppressors may lead to better understanding of signal transduction downstream of RLKs.

## MATERIALS AND METHODS

### Plant Growth Conditions and Mutant Screens

All plants were grown under 16 h light at 23°C and 8 h dark at 20°C. To screen for mutants with *snc1*-like constitutive defense responses, wild-type Col-0 seeds were mutagenized with EMS and the M2 progeny were analyzed for enhanced resistance to *H. a. Noco2*. *snc4-1D* was one of mutants identified. To search for suppressors of *snc4-1D*, seeds of *snc4-1D* were obtained by growing the plants at 28°C and mutagenized with EMS. About 12,000 M2 plants were screened to identify mutants that can grow to maturity and set seeds at 22°C.

### Mutant Characterization

Two-week-old seedlings grown on one-half-strength Murashige and Skoog medium were used for gene expression analysis, trypan blue, and DAB staining. For gene expression analysis, RNA was extracted using Takara RNAiso reagent (Takara) and RT was performed using the Takara M-MLV RTase cDNA synthesis kit. Real-time PCR was carried out with the SYBR Premix Ex (Takara). The primers used for amplification of *Actin1*, *PR1*, and *PR2* were described previously (Zhang et al., 2003b). The primers used for amplification of *PDF1.2* are PDF1.2-F (5'-atcacccttatctcgtcgc-3') and PDF1.2-R (5'-tgctgggaagacatagttgc-3'). Trypan blue and DAB staining were carried out as previously described (Parker et al., 1996; Thordal-Christensen et al., 1997). Analysis of resistance to *H. a. Noco2* was performed by spraying 2-week-old seedlings with *H. a. Noco2* spores ( $5 \times 10^4$  spores/mL). For each genotype, seedlings grown in three different pots (15 seedlings per pot) were inoculated. Seven days post inoculation, plants from each pot were collected as one sample and total number of conidia spores for each sample was counted with a hemocytometer.

### Map-Based Cloning of *snc4-1D*

Positional cloning of *snc4-1D* was performed according to procedures previously described (Zhang et al., 2007). The markers used to map *snc4-1D* were derived from insertion-deletion and single nucleotide polymorphisms identified from the genomic sequences of Col-0 and *Ler* ecotypes provided by Monsanto (Jander et al., 2002). The primer sequences for F13O11 are 5'-cac-atcttcagacactgccac-3' and 5'-gcttgaatctgctgttc-3'. Primers for F20P5 are 5'-agtcctgtttacatgctc-3' and 5'-tggaactggcgcttctgtc-3'. These two markers are based on insertion-deletion polymorphisms. For marker T4O24 that detects single nucleotide polymorphism, primers 5'-ctttctaagctcagtaacagg-3' and 5'-gaccgttatgcacgtaaatg-3' were used to detect the presence of the Col-0 allele and primers 5'-ctttctaagctcagtaacagg-3' and 5'-gaccgttatgcacgtaaatg-3' were used to detect the presence of the *Ler* allele. For marker F1O19, DNA was

amplified by PCR using primers 5'-cttctgtgtctcaccggtg-3' and 5'-tttggagccgg-tatcaatgg-3' and sequenced to detect the polymorphisms in Col-0 and *Ler*.

### Plasmid Construction and Arabidopsis Transformation

For complementation analysis, a 6.6-kb genomic DNA fragment containing *SNC4* or *snc4-1D* was amplified by PCR using primers 5'-acgcacgctcagc-cttgacacaagaggtacag-3' and 5'-cggggatccgagctcgaatctttagggccttgg-3' and cloned into the binary vector pGREEN229 (Hellens et al., 2000). To confirm that the phenotypes observed in *snc4-1D* were caused by the mutation in *snc4-1D*, pGREEN229-SNC4 and pGREEN-snc4-1D were transformed into *Agrobacterium* and subsequently into the wild-type plants by floral dipping (Clough and Bent, 1998). Transgenic plants were identified by Basta selection and confirmed by PCR.

### Localization Assay in Protoplast

For localization analysis, the full-length cDNA fragments of *SNC4* and *snc4-1D* were amplified by PCR using primers SNC4-GF (5'-cggggatccgagctcgaatctcagcaaaagtagac-3') and SNC4-GR (5'-acgcacgctcagcactcttctgttagacagagac-3') and cloned into a modified pCAMBIA1300 vector containing an in-frame GFP tag at the C terminus. The pCAMBIA1300-GFP-SNC4 and pCAMBIA1300-GFP-snc4-1D were transformed into protoplast as described (Sheen, 2001) and examined by confocal microscopy.

### Creating the Double Mutants

Since *snc4-1D* plants cannot grow to maturity, the *snc4-1D/SNC4* heterozygous plants were crossed with homozygous *opr3-2*, *eds1-2*, *ndr1-1*, and *mks1-1* (GT\_5\_108403) to generate various double mutants. F1 plants heterozygous for *snc4-1D* were identified by PCR. All the double mutant plants were identified in the F2 progeny by PCR. *eds1-2* and *ndr1-1* were described previously (Century et al., 1995; Parker et al., 1996). The *eds1-2* line used was introgressed into Col by multiple backcrossing and was kindly provided by Dr. Jane Parker. *opr3-2* was kindly provided by Dr. Jianmin Zhou, and it contains a mutation in *OPR3* that creates an early stop codon in the cDNA. The *mks1-1* mutant is in *Ler* background and it is closely linked to the *RPP5* locus. The *snc4-1D mks1-1* double mutants were therefore identified for the presence of the *RPP5* locus (from *Ler*) and two independent lines with *RPP5* absent were used for *H. a. Noco2* infection experiments.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number HM461758.

### Supplemental Data

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

**Supplemental Figure S1.** Analysis of the size of SNC4 cDNA and SNC4-GFP fusion protein.

**Supplemental Figure S2.** Localization of SNC4-GFP and *snc4-1D*-GFP fusion protein.

**Supplemental Figure S3.** Trypan blue staining (top section) and DAB staining (bottom section) of true leaves of wild type, *snc4-1D*, WT::*SNC4* line 1, and WT::*snc4-1D* line 1.

**Supplemental Figure S4.** Sequence alignment of the extracellular domain of SNC4 and six GDPD domain-containing proteins in Arabidopsis.

**Supplemental Figure S5.** Western-blot analysis of SNC4 protein levels in wild type (WT), *snc4-1D*, and *snc4-4* using a polyclonal anti-SNC4 antibody generated against a fragment of the SNC4 kinase domain.

**Supplemental Figure S6.** A working model for SNC4-mediated resistance.

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