Mutagenic Analysis of Victorin Sensitivity in *Arabidopsis thaliana* and *Avena sativa*

by
Rishi Sharma Seshadri

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(Honors Scholar)

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Presented December 5, 2018
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Abstract approved:_____

Thomas Wolpert

The fungal pathogen *Cochliobolus victoriae*, the causal agent of Victoria blight, produces a host-selective pathogenicity factor called victorin. In *Arabidopsis thaliana*, a dominant gene called "supersense" (*SSN*) confers constitutive victorin sensitivity at very low concentrations. *SSN* encodes a nucleotide-binding leucine-rich-repeat (NB-LRR) protein. In the *SSN*-carrying Heiligkreuztal 2 ecotype (TAIR Germplasm Stock CS76404) of *Arabidopsis thaliana*, a mutagenic approach was undertaken to reveal the presence of second-site mutations conferring insensitivity to victorin. Genetic analysis identified several mutants possessing a functional *SSN* allele while displaying insensitivity to victorin. These data suggest that at least one additional gene is required for *SSN* gene function and expression of the victorin super-sensitive phenotype.

Previous studies in *Avena sativa* indicate that Victoria blight susceptibility, conferred by the *Vb* gene, and crown rust resistance, conferred by the *Pc2* gene, are genetically inseparable, suggesting that these distinct responses may actually be controlled by the same gene. We undertook a mutagenic analysis of the victorin-sensitive

Victoria cultivar to assess the correlation between these distinct disease phenotypes. In nearly all victorin-insensitive mutants identified, loss of sensitivity to victorin also resulted in the complete loss of resistance to rust. This finding supports the assertion that *Vb* and *Pc2* are the same gene and *Vb/Pc2* is responsible for both victorin sensitivity and rust resistance. These responses are likely mediated by an NB-LRR protein.

Key Words: victorin, Victoria blight, crown rust, NB-LRR, second-site mutation

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Honors Baccalaureate of Science in Microbiology and Honors Baccalaureate of Arts in Music project of Rishi Seshadri presented on December 5, 2018.

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Rishi Sharma Seshadri, Author

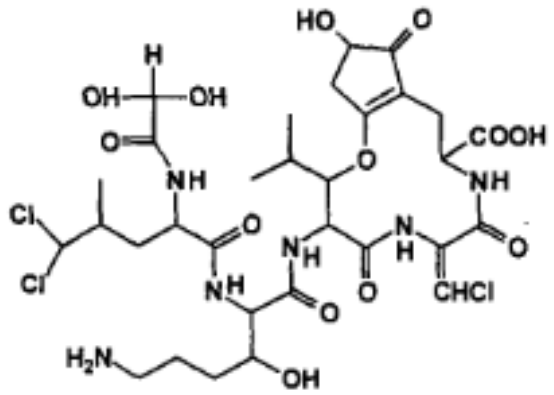
Introduction

Fungal pathogens are responsible for large amounts of crop destruction and food spoilage around the world. In the 1940s, the fungal pathogen *Cochliobolus victoriae*, the causal agent of Victoria blight, caused severe damage to Victoria-related oat varieties in the United States. These Victoria oat varieties were widely planted because they possess the *Pc-2* gene that confers resistance to *Puccinia coronata*, the causal agent of oat crown rust. At the time, nearly 80% of oat varieties in the United States were derived from Victoria, resulting in massive yield losses due to the rapid spread of *C. victoriae.* Interestingly, efforts to genetically separate Victoria blight susceptibility and rust resistance have been unsuccessful, suggesting that oat crown rust resistance and Victoria blight susceptibility are controlled by the same gene.

C. victoriae produces a pathogenicity factor called victorin. This host-selective toxin elicits cell death in the host (Meehan & Murphy, 1946) and facilitates virulence of the necrotrophic fungus. The presence of a single dominant gene, *Vb*, confers sensitivity to victorin and thus susceptibility to the fungus. *C. victoriae* appears to use the host's innate immune defenses for successful infection. While the pathogen causes disease in the host, it stimulates a response resembling that of disease resistance. Such symptoms include callose production, a respiratory burst, ethylene evolution, extracellular alkalinization, and K+ ion efflux (Ullrich et al., 1991; Walton et al., 1985). All of these symptoms are associated with the defense response.

In Victoria Blight susceptible hosts, *C. victoriae* also provokes a programmed cell death (PCD) response, which is another response normally associated with defense.

Ordinarily, the resistance-associated hypersensitive response (HR), a type of programmed cell death response, quarantines an invading biotrophic pathogen. This response involves the rapid death of cells localized to the infection site, restricting the spread of the pathogen to other parts of the plant. However, *C. victoriae* is undeterred by HR-related cell death because, as a necrotrophic fungus, it feeds on dead tissue. The HR instead allows for rapid colonization of the host by *C. victoriae* (Govrin & Levine, 2000).



Victorin, the pathogenicity factor produced by *Cochliobolus victoriae*

Plant immune responses comprise two broad categories. The first level primarily involves pattern recognition receptors (PRRs) recognizing evolutionary conserved pathogen-associated or microbe-associated molecular patterns (PAMPs & MAMPs). The second level of immunity involves R proteins mounting effector-triggered responses. R proteins are encoded by resistance genes (R genes) and are typically nucleotide-binding, leucine-rich-repeat proteins (NB-LRRs). Generally, R proteins yield a resistance phenotype via an interaction with pathogen effectors. Pathogen effectors promote virulence by interfering with PRR-mediated defense. However, when effectors are recognized by R proteins and initiate defense, the effectors are called avirulence (*Avr*)

products. The structures and host interactions of *Avr* effectors vary widely, which is likely the result of co-evolutionary mechanisms which put pressure on pathogens to avoid recognition by their host.

The relationship between R genes and *Avr* genes, first described by Flor, is known as gene-for-gene resistance (Flor, 1971). The gene-for-gene relationship requires (1) the production of a specific R gene product that confers resistance to a pathogen expressing (2) a corresponding *Avr* gene product (effector). Crown rust of oats exemplifies a typical gene-for-gene resistance model. Oats maintain dominant R genes that match dominant *Avr* genes from *P. coronata*. R genes typically encode receptors (NB-LRRs) specific to certain effectors. Thus, the suite of R proteins present in the host allows for recognition of numerous pathogen effectors. In oats, the dominant *Pc2* gene (likely encoding an NB-LRR resistance protein) confers resistance to *P. coronata* race 203 via recognition of the AvrPc2 effector (Figure 1).

Figure 1: Classic gene-for-gene model of plant immunity

		Host Resistance Gene (e.g. *Pc2*)	
		R-	*rr*
Pathogen Effector Gene (e.g. *AvrPc2*)	*A-*	Resistant	Susceptible
	aa	Susceptible	Susceptible

In contrast with the model for crown rust, Victoria blight conforms to an inverse gene-for-gene resistance model. Under this model, the presence of dominant *vir* (virulence) and S (susceptibility) genes results in host susceptibility (Fenton, 2009). Only in the presence of homozygous recessive genes for either of these factors does resistance occur (Figure 2). With respect to Victoria blight, victorin acts as the virulence effector. The S gene (*Vb*) is likely an NB-LRR that possibly shares identity with the host R gene for crown rust (*Pc2*), a claim which is investigated in this study.

Figure 2: Inverse gene-for-gene model of plant immunity

		Host Susceptibility Gene (*e.g. Vb*)	
		S-	ss
Pathogen Effector Gene (e.g. victorin)	E-	Susceptible	Resistant
	ee	Resistant	Resistant

In *Arabidopsis thaliana*, the locus orchestrating victorin effects (*LOV1*) was identified. This was the first gene conditioning victorin sensitivity that was molecularly characterized and the first gene for victorin sensitivity identified in *Arabidopsis*. Analogous in function to *Vb*, *LOV1* yields a susceptibility phenotype when inoculated with *C. victoriae*. Interestingly, victorin sensitivity regulated by this gene displays a developmental phenotype where older plants are more sensitive to the toxin than younger plants. (Lorang *et al.*, 2004; Lorang *et al.*, 2007). The *LOV1* gene encodes a protein

belonging to NB-LRR family. NB-LRR proteins are frequently implicated in pathogen detection and disease resistance (DeYoung & Innes, 2006). Thus, these findings in Arabidopsis support the possibility that *Vb* and *Pc2* in oats may be the same gene encoding an NB-LRR.

A second gene is also necessary for victorin sensitivity conferred by *LOV1* (Sweat & Wolpert, 2007). A disruption of this gene, *TRXh5*, encoding thioredoxin-h5 (TRXh5), causes a loss of victorin sensitivity. Thus, TRXh5 is a second site locus where both *LOV1* and *TRXh5* are necessary to condition the victorin response. Thioredoxins are a class of small proteins present in all organisms. As the name implies, these proteins are usually involved in redox regulation, including the resolution of reactive oxygen/nitrogen species, regulation of protein oxidation state, and signaling regulation. TRXh5, which is required for victorin sensitivity, is one of eight distinct “h-type” thioredoxins. While members of this class of thioredoxins tend to exhibit some degree of functional redundancy, TRXh5 is predominantly associated with defense. Victorin covalently binds to TRXh5. Data form earlier studies show that LOV1 associates with ("guards") TRXh5 in the absence of victorin, but the binding of victorin to TRXh5 triggers LOV1 to disassociate and signal the HR. Therefore, binding of the victorin toxin to TRXh5 ultimately results in host susceptibility to *C. victoriae* (Lorang, *et al.*, 2007).

Aside from the response conditioned by *LOV1* and *TRXh5*, another independent NB-LRR encoding gene has been discovered and characterized in Arabidopsis that confers victorin sensitivity. The super-sensitive gene (supersense, *SSN*) conditions a response at very low concentrations of toxin (on the order of nM). Unlike *LOV1*, which is developmentally regulated, the supersense gene confers constitutive sensitivity to

victorin. Hence, *Arabidopsis* plants that possess a functional allele at the *SSN* locus display "super-sensitivity" to the toxin. Like *LOV1*, supersense segregates as a dominant gene. Again, the association of another NB-LRR-encoding gene with victorin sensitivity suggests that victorin sensitivity is always encoded by NB-LRRs. Moreover, *Vb* and *Pc2* in oats may actually be the same NB-LRR-encoding genes.

Direct identification of an NB-LRR-encoding gene in oats poses significant challenges due to the large size and limited mapping of the allohexaploid genome. As a substitute, genetically tractable species have been interrogated to infer the role of an NB-LRR as the agent conditioning victorin sensitivity. In barley, another cereal, developmental sensitivity to victorin was identified in chromosome-doubled haploid lines of the Baronesse cultivar (Lorang *et al.*, 2010). A locus responsible for victorin sensitivity was identified and localized to an R-gene-rich region of the barley genome, suggesting that an R gene is indeed responsible for victorin sensitivity. (Lorang *et al.*, 2010)

This investigation had two discrete aims. With respect to oats, our objective was to assess the correlation between rust resistance and victorin sensitivity, given the preponderance of evidence suggesting that the *Pc-2*/*Vb* genes are one and the same. If loss of victorin sensitivity also confers a loss of rust resistance, this would strongly suggest the two genes are the same. Consistent with the role of NB-LRRs in disease resistance and findings in various other plants, this result would also suggest that *Vb* likely encodes an NB-LRR protein. We interrogated this hypothesis using a mutagenic analysis of the Victoria cultivar of oats, which typically displays resistance to *P. coronata* and susceptibility to *C. victoriae*. Mutagenized plants were initially screened for victorin

insensitivity and subsequently inoculated with *P. coronata* race 203 to determine whether both phenotypes are conditioned by the same *Pc-2*/*Vb* gene.

Secondly, we used a mutagenic approach to evaluate the presence of second-site mutations conferring victorin insensitivity in the Heiligkreuztal 2 (HKT2.4) ecotype of *Arabidopsis*, which carries the dominant supersense (*SSN*) gene. As with oats, we screened mutagenized seed for victorin insensitivity. Genetic analyses of identified victorin-insensitive mutants were carried out to infer whether loss-of-function mutants were sustained in the supersense gene or at a second locus. The purpose of this portion of the investigation was to examine whether *SSN* has similar functional requirements as *LOV1* and to perhaps, by inference, illuminate requirements for *Vb/Pc2* function in oats.

Materials & Methods

Growth Conditions

Unless otherwise noted, routine growth of *A. thaliana* plants and *A. sativa* seedlings was conducted in a controlled indoor growth chamber at 25°C under a 16-hour light/8-hour dark cycle. After approximately two to three weeks of indoor growth, oats were transferred to a greenhouse facility for seed production.

Arabidopsis thaliana Mutant Production and Screen

Mutagenesis of *A. thaliana* was performed as previously described (Sweat and Wolpert, 2007) with seed from the HKT2.4 ecotype (TAIR Germplasm Stock CS76404). M2 seed were generated by planting mutagenized M1 seed in 4" pots at approximately 25 seeds/pot. Seed was bulk collected from each pot and maintained as separate stocks for screening. 239 stocks were collected for screening.

Mutants were selected on the basis of their insensitivity to victorin. 100 mg of seed (approximately 4000 seeds) from each stock was sterilized with 500 uL of 70% ethanol for five minutes. After removing the ethanol solution, seeds were rinsed with 500 uL of 20% bleach solution and incubated for five minutes. This bleach solution was diluted with 1 mL of water, then removed. Finally, seeds were washed three times with sterile, deionized water. After washing, the seed was stratified at 4°C for five days in 200 uL of sterile, deionized water before plating on 55 X 5 mm plates containing 5ml 0.8% agarose. Immediately prior to seed plating, agarose plates were spread with 100 µl of sterile water containing 500 ng of victorin. Five to seven days after plating, plants that grew on the agarose plate were transplanted to soil.

If only a single plant was viable in the stock, this surviving plant was assumed to be escapes. However, if two or more plants grew on the agarose plate from a single stock, then these siblings were assumed to be mutants. Mutant progeny were confirmed by infiltration of victorin according to the *A. thaliana* leaf infiltration bioassay below to confirm the insensitive phenotype.

A. thaliana Leaf Infiltration Bioassay

A 1 mL syringe was used to infiltrate victorin solution into leaves of *A. thaliana*. Solution was injected into the underside of an accessible leaf and marked with a black marker. Sufficient solution was injected to allow it to visibly spread through the entire leaf. *A. thaliana* leaves were infiltrated with victorin concentrations of 1-10 μg/mL. Phenotypes of marked leaves were scored 24 hours after initial infiltration.

Arabidopsis thaliana Crosses

To prepare for the genetic cross, unopened flowers of the pollen recipient were emasculated, leaving the pistil exposed. In the donor flower, pollen was exposed by gently pinching the flower by hand near the receptacle/peduncle. To complete the cross, exposed pollen from the donor flower was gently brushed against the recipient pistil to deposit pollen on the stigma. An emerging silique became visible within a few days following a successful cross.

Avena sativa Mutant Production and Screen

Mutagenesis of *Avena sativa* was performed as previously described (Chawade *et al*., 2010) with seed from the Victoria cultivar. Mutagenized seed was planted in 4" pots (30-40 seeds per pot) and placed in a growth chamber at 25°C under a 16-hour light/8-hour dark cycle. Approximately 1 week after germination, individual seedlings were transplanted to 6.5 X 25 cm planting cones and arranged in racks with 12 cones/seedlings per rack. Plants were grown in the greenhouse until seed had matured and seed was bulk collected from each rack and maintained as separate stocks (i.e. each stock contained seed from 12 plants) for screening.

Mutants were initially selected based on their response to inoculation with *Cochliobolus victoriae*. Seed from each stock was planted in 10 × 20 × 2.5 inch flats and incubated in a growth chamber at 25°C under a 16-hour light/8-hour dark cycle for approximately 10 days. Flats were inoculated by spraying 50-75 ml of a 10^5 spores/mL suspension of *C. victoriae* in 0.01% Tween-20 solution. Inoculated plants were covered with clear plastic lids. and sealed around the base of the lid with masking tape to maintain humid conditions. Trays were incubated for three days to ensure favorable infection conditions. Subsequently, trays were open to the air for three days before undergoing a second round of inoculation. Remaining viable plants, typically 0-3 plants per flat, were infiltrated with 0.5-1 mg/mL victorin solution to confirm the insensitive phenotype. After at least two weeks of growth in the indoor growth chamber, these viable plants were transplanted into separate six-inch diameter pots and incubated in the greenhouse. Multiple screened plants selected from the same flat were considered siblings.

Cochliobolus victoriae Spore Production

This assay selected for victorin-insensitive oats from a mixed population. 100 µL of *Cochliobolus victoriae* spores from stocks stored in 20% glycerol at -80° C were plated onto V8 agar and incubated for 10 days at 25° C in a 12-hour light/dark cycle to promote spore development. After incubation, spores were scraped from the plates and suspended in 30 mL 0.01% Tween-20 solution, counted with a hemocytometer and the volume adjusted to a spore concentration of 10^5 spores/mL.

Using a handheld sprayer, the suspended spores were sprayed onto the 10-day-old oat population of interest. Four flats of oats could be treated with 200-300 mL of the suspended spores. Each flat of oats was sealed for three days to maintain favorable infection conditions. Flats were unsealed and left open to ambient air for three days before undergoing a second inoculation. The remaining viable oats were infiltrated with 1 µg/mL victorin solution to confirm the victorin insensitive phenotype.

Infiltration of Victorin in *Avena sativa*

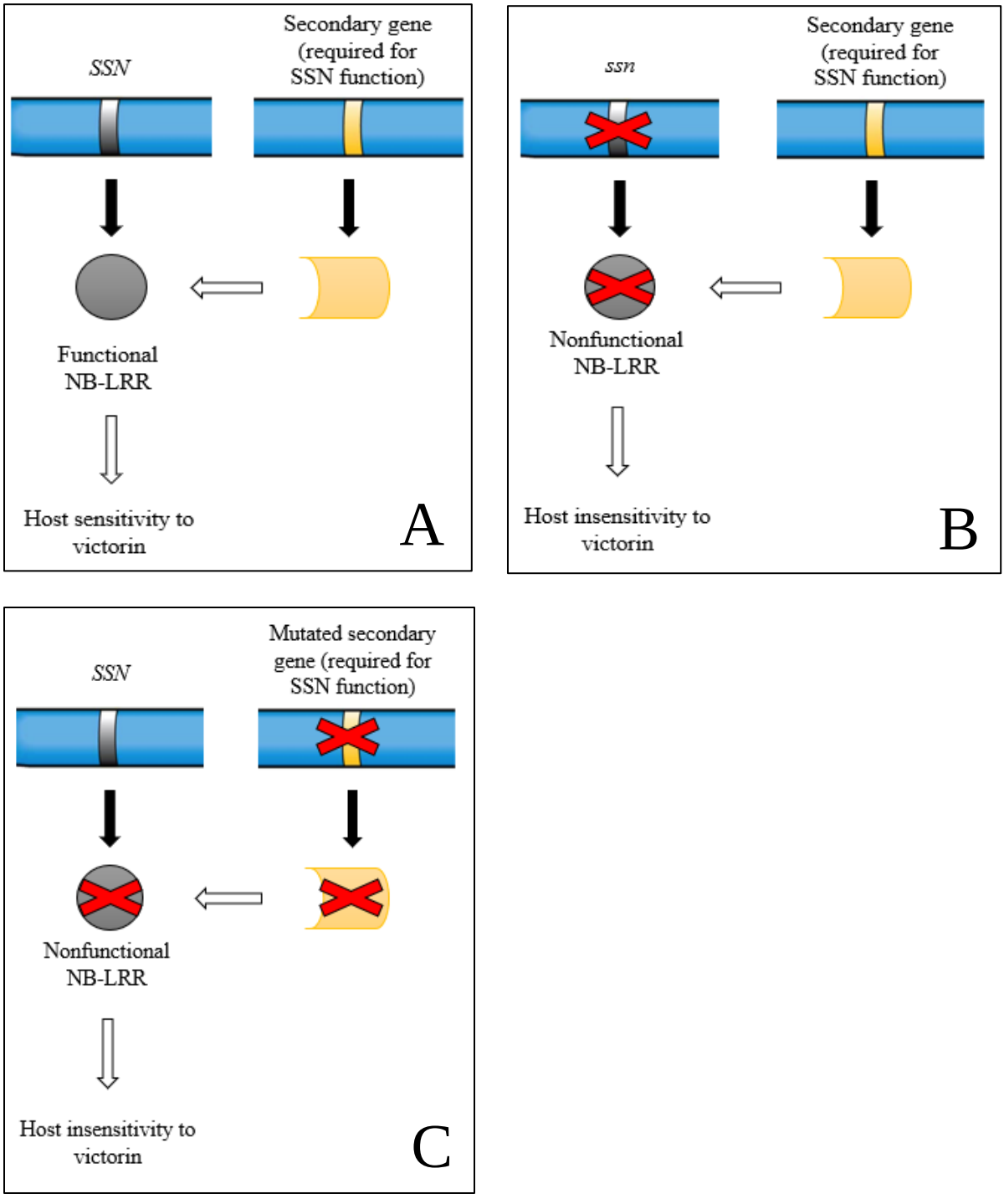
In oats, victorin infiltration was carried out using a needleless 1 mL syringe. A one-inch-wide space was marked three inches from the tip of the youngest primary leaf on the main stem. Sufficient solution was injected such that it spread through the entire inch-wide space that was previously marked. Oats were infiltrated with various victorin concentrations of 0.5-10 µg/mL to confirm the strength of the phenotype.

Results

Arabidopsis thaliana

239 pools of seed, representing approximately 6,000 mutant plants, were prepared from the EMS-treated HKT2.4 ecotype and screened for survivors by planting 1 mg of seed (about 4000 seeds) from each pool on an agarose plate with 200ng of victorin for five days. From this screen, we initially identified 19 victorin-insensitive *Arabidopsis* mutant populations as carrying a potential mutation of interest. The dominant supersensitive gene, which confers constitutive sensitivity to victorin, is present in the HKT2.4 ecotype. Victorin-insensitive *Arabidopsis* mutant lines arose most likely due to either (1) a loss-of-function mutation in the supersensitive gene itself or (2) a mutation in a gene at a locus different from the supersensitive locus but required for supersensitive gene function (Figure 3). This study was concerned only with second-site mutations, not mutations in the super-sensitive gene itself.

Figure 3: Likely mutagenic outcomes, including (A) functional SSN and secondary protein yielding victorin sensitivity, (B) *ssn* mutant which is insensitive to victorin, or (C) second-site mutant which is insensitive to victorin



Mutant HKT2.4 carries the functional super-sensitivity allele and a second-site mutation rendering the *ssn* gene nonfunctional. In contrast, Columbia carries the nonfunctional super-sensitivity allele and presumably the wildtype allele at the second site of interest. Each of the 19 mutants displayed insensitivity to the toxin up to a concentration of at least 10 µg/mL (highest concentration tested). For further genetic analyses, each mutant line was grown to maturity and crossed to the victorin-insensitive Columbia ecotype as the maternal parent (mutant HKT2.4 × Col-0). Because the Columbia ecotype is homozygous recessive at the supersensitive locus, a cross with the HKT2.4 mutants could reveal the presence of recessive second-site mutations. If the gene for super-sensitivity was unaffected by mutagenesis, then the mutant HKT2.4 x Col-0 F1 hybrid would be heterozygous for both the dominant supersensitive gene and any recessive second-site mutation with the latter being complemented by the Col-0 genetic contribution. Consequently, the F1 hybrids would express the supersensitive phenotype.

Alternatively, if the super-sensitivity gene was mutated, and because the Columbia ecotype is homozygous recessive at the supersensitive locus, the mutant HKT2.4 x Col-0 F1 hybrid would carry nonfunctional *ssn* alleles and be insensitive. Therefore, we inferred that insensitive F1 hybrids likely inherited a non-functional supersensitive allele from the HKT2.4 mutant parent, indicating that the mutant parent contained a loss-of-function mutation in the super-sensitivity gene rather than a second-site mutation. Because only second-site mutants were being interrogated, only victorin-sensitive progeny of the mutant HKT2.4 × Col-0 hybrids were analyzed.

In the F1 generation, 15 of the 19 total mutagenized parents yielded victorin-insensitive progeny, indicating that these lines carried a mutation in the supersense gene rather than a separate gene of interest. Therefore, we did not conduct further analysis of these 15 lines. The remaining four mutant HKT2.4 x Col-0 F1 progeny derived from the mutant parents, designated as HKT2.4-73, HKT2.4-181, HKT2.4-201 and HKT2.4-209, produced toxin-sensitive individuals. These victorin-sensitive hybrids indicated potential second-site mutations because they retained the dominant supersensitive phenotype (displaying constitutive sensitivity to victorin at low concentrations), indicating that mutagenesis did not affect the functionality of the supersense allele.

To facilitate further genetic analyses, victorin-sensitive F1 individuals were allowed to self-fertilize and seed were collected. F2 progeny populations were scored to discern the segregation ratio for victorin sensitivity versus insensitivity, which can be used to infer the presence of second-site mutations (Table 1). The Pearson chi-squared goodness of fit test was used to evaluate the statistical significance of the F2 segregation data (Mantel, 1963). The chi-squared test is used to determine whether a specific observable outcome in a population could plausibly be attributed to chance. In statistical terms, the p-value of a data set is the probability of obtaining a random sample as extreme as the observed sample relative to the null hypothesis (Lock *et al.*, 2013) The null hypothesis for this test was calculated with an S:I ratio of 9:7, the most likely phenotypic segregation ratio implied by the segregation data. In this case, the chi-square test fails to reject the null hypothesis that the population segregates in a 9:7 ratio with respect to victorin sensitivity.

Table 1: F2 segregation analysis of victorin sensitivity for progeny of four HKT2.4 mutants

Cross (Donor × Recipient)	F2 Segregation (S:I)[a]	F2 Total Progeny (*N*)	Percentage of Sensitive Progeny	Two-tailed p-value[b]
HKT2.4-73 × Col-0	163:110	273	60%	0.2496
HKT2.4-181 × Col-0	193:144	337	57%	0.7058
HKT2.4-201 × Col-0	163:113	276	59%	0.3470
HKT2.4-209 × Col-0	159:115	274	58%	0.5527

[a]I denotes victorin insensitivity, S denotes victorin sensitivity
[b]calculated using Pearson's chi-squared test

This segregation ratio in Table 1 suggests that a second functional gene is necessary for expression of the victorin super-sensitive phenotype. If a second gene was not required, we would not have found sensitive F1 individuals and would have observed an F2 population consisting of only insensitive individuals. However, we observed a 9:7 (S:I) phenotypic segregation. This is because ¼ of the progeny carrying the dominant supersense allele are also homozygous recessive at a second-site mutation. Therefore, 1/4 of the 3/4 that would normally be sensitive because of the supersense gene are rendered insensitive. Consequently, only 9/16 (9:7 segregation ration) of the population are expected to be sensitive if a second-site recessive mutation exists (Table 2).

Table 2: Progeny of mutant HKT2.4 x Columbia cross

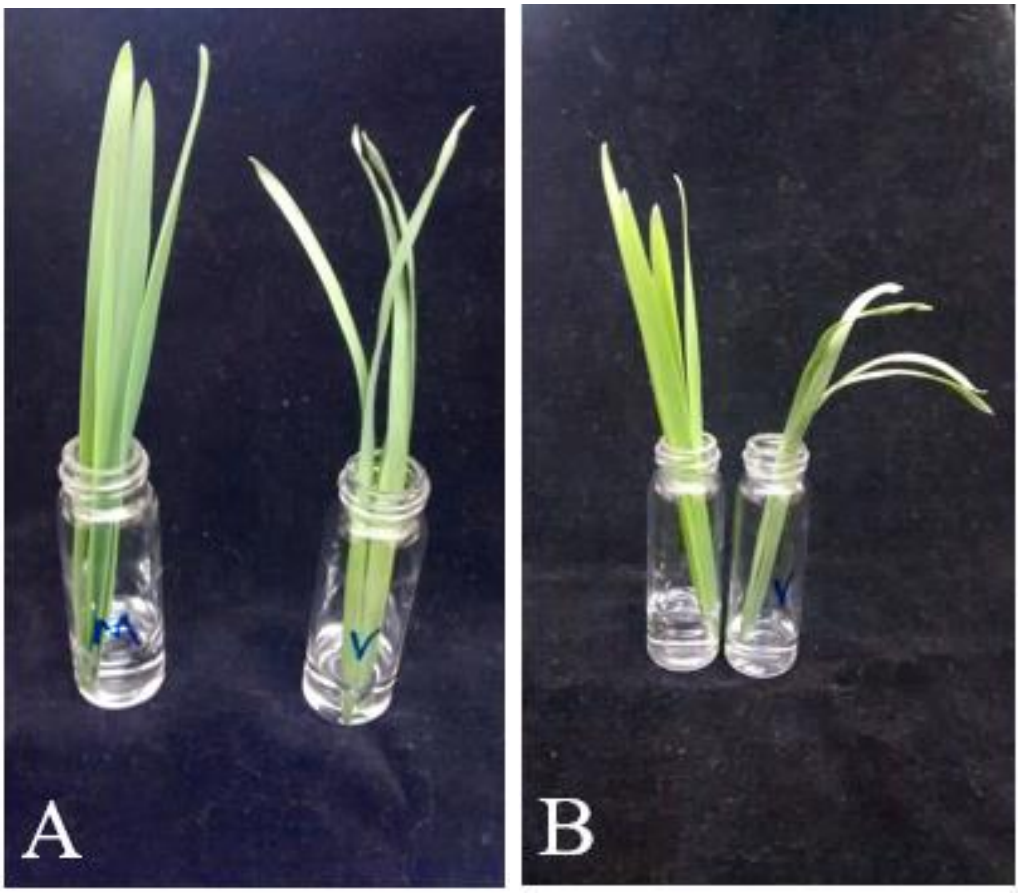
Possible F2 Genotypes				
	SA	**Sa**	**sA**	**sa**
SA	SSAA	SSAa	SsAA	SsAa
Sa	SSAa	SSaa	SsAa	Ssaa
sA	SsAA	SsAa	ssAA	ssAa
sa	SsAa	Ssaa	ssAa	ssaa

[1]Green cells denote victorin-insensitive progeny, yellow cells indicate victorin-insensitive second-site mutants, and red cells indicate victorin-sensitive progeny.

Avena sativa

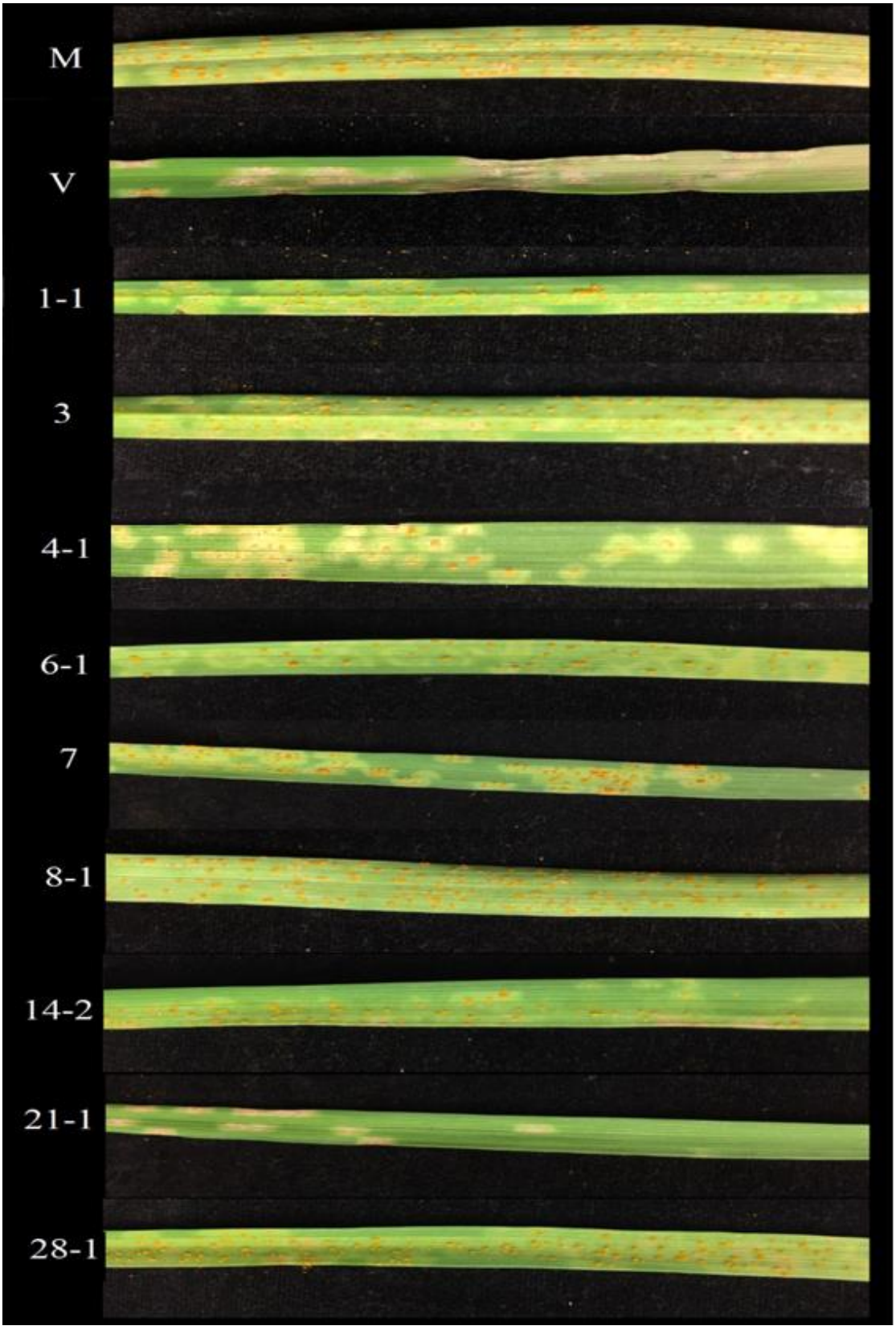
Mutant oats were identified by inoculation with *C. victoriae* on the basis of their resistance to Victoria blight. These oats were grown to maturity and allowed to self-fertilize. The subsequent generation (M2) was grown and inoculated with *P. coronata* to identify the responses to rust. These same plants were also re-screened via direct infiltration of victorin (1 ug/mL) and a detached leaf assay (1 µg/mL) to confirm loss-of-function in the *Vb* gene. Wild-type Marvelous and Victoria cultivars were used as negative and positive controls for victorin sensitivity respectively. Phenotypic response to victorin is shown (Figure 4).

Figure 4: Insensitive (left) and sensitive (right) responses to 1 µg/mL victorin solution using detached leaf assay after 24 hours (A) and 48 hours (B)



Each mutant was also inoculated with spores from *P. coronata* race 203 to determine whether mutations yielding insensitivity to victorin also affected the response to rust. Rust-susceptible mutants displayed slight chlorosis surrounding distinctively orange-colored pustules (uredinia) on the leaf blades. Powdery, rust-colored urediniospores are also visible. Notably, the Victoria-resistant mutant 21-1 displays tissue necrosis surrounding infection sites and uredinia are absent. On the 4-1 mutant, small uredinia are surrounded by necrotic regions. Rust responses are shown (Figure 5).

Figure 5: Mutant responses to inoculation with *P. coronata*



The data from the assays for victorin and rust phenotypes are summarized in Table 6. We identified nine victorin-insensitive, Victoria blight-resistant mutants eight of which were also rust susceptible. However, rust inoculation assays indicated that the victorin insensitive mutant 21-1 was different from the other mutants, displaying both resistance to *P. coronata* and resistance to *C. victoriae*.

Table 6: Phenotypes of Mutant Victoria Oats

Oat Type	**Response to P. coronata**	**Response to C. victoriae**
Marvelous	S	R
Wild-type Victoria	R	S
Mutant Victoria 1-1	S	R
Mutant Victoria 3	S	R
Mutant Victoria 4-1	weakly S	R
Mutant Victoria 6-1	S	R
Mutant Victoria 7	S	R
Mutant Victoria 8-1	S	R
Mutant Victoria 14-2	S	R
Mutant Victoria 21-1	R	R
Mutant Victoria 28-1	S	R

[a]I denotes insensitivity, R denotes resistance, S denotes susceptibility
[b]Wild-type Marvelous- and Victoria-type oats were included as negative and positive controls for phenotype scoring

Discussion

The aims of this investigation were two-fold. First, we sought to identify second-site mutants of *Arabidopsis* in the victorin-supersensitive HKT2.4 ecotype to gain insights into the molecular mechanism governing *SSN*. Ethyl methanesulfonate (EMS) mutagenesis was used to prepare approximately 6000 mutagenized plants whose progeny were screened for victorin-insensitivity. In total, loss-of-sensitivity mutants were identified in 19 seed populations. Because this study was concerned only with second-site mutations, mutants were crossed to the victorin-insensitive Columbia ecotype (Col-0), which possesses a nonfunctional *ssn* gene. The genetic analysis indicated that, of the 19 populations initially identified, four likely possessed a loss-of-function mutation in a gene distinct from *SSN* resulting in insensitivity to victorin. Backcrosses with Col-0 revealed that the other 15 victorin-insensitive mutants carried a loss-of-function mutation in the *SSN* gene itself.

These results provide greater insight into the genetic factors conditioning the victorin response. The major finding from this part of our investigation is evidence of an independent gene whose function is required for proper expression of the supersense phenotype. A model describing the molecular response to victorin has already been established with respect to the developmental victorin-sensitivity gene, *LOV1* (Lorang *et al*., 2007). In the case of *LOV1*-conditioned victorin sensitivity, victorin-mediated activation of the LOV1 NB-LRR protein provokes a defense-associated HR and cell death in the host. In addition to LOV1, expression of thioredoxin h5 (ATTRX5) is required for victorin sensitivity (Sweat & Wolpert, 2007). However, the requirement for ATTRX5 can be partially compensated by overexpression of thioredoxin h3 (ATTRX3).

While different thioredoxin proteins likely carry out distinct cellular roles, unique functions for each have not been identified (Åslund & Beckwith, 1999). In various hosts, partially or completely redundant functions have been observed across the thioredoxin family (Laloi *et al.*, 2004). Previous bioinformatic analyses confirm that *SSN* encodes an NB-LRR protein, as is the case with *LOV1*, and is responsible for the supersensitive phenotype. Unpublished results indicate that also similar to *LOV1*, a thioredoxin is required for SSN function but that either ATTRX5 or ATTRX3 can support *SSN* function, but this requirement is not yet unambiguous. Because simultaneous occurrence of an attrx-h5/attrx-h3 double mutant is a highly unlikely outcome of mutagenesis, we posit that the identity of the second site gene identified in this study may instead be a chaperone protein.

There are several known chaperone proteins which are required for NB-LRR function. Without the development of recognition competence, NB-LRR proteins are unable to function properly to recognize pathogen-derived effector molecules. Competent NB-LRRs must be correctly folded and held in a suitable cellular location to facilitate immune signal transduction (Shirasu, 2009). While the mechanisms that underlie NB-LRR processing are not fully understood, there are three key proteins that are known to stabilize NB-LRR proteins: HSP90, RAR1, and SGT1 (Shang *et al.*, 2006).

Firstly, heat shock protein 90 (HSP90) is a highly conserved molecular chaperone protein that, among other functions, is critical for the development of the proper conformation of many proteins (Pearl *et al.*, 2008). In *Arabidopsis*, four distinct HSP90 (*HSP90.1*, *HSP90.2*, *HSP90.3*, and *HSP90.4*) proteins may be present in the cytoplasm

(Takahashi *et al.*, 2003). Loss-of-function mutations in *HSP90.1* and *HSP90.2* result in compromised resistance phenotypes (Staal *et al.*, 2006; Takahashi *et al.*, 2003).

Secondly, RAR1 has also been identified as a HSP90 interacting partner whose expression is required for NB-LRR function (Top & Jørgensen, 1986). Several independent studies of *rar1* mutants in *Arabidopsis* suggest that the protein is responsible for the proper function of several NB-LRR proteins, including those recognizing effectors of *Hyaloperonospora arabidopsis*, *Leptosphaeria maculans*, and *Pseudomonas syringae*. (Shirasu, 2009). *RAR1* is not known to play a role in plant development and its functions are apparently limited to NB-LRR-associated immunity. Indeed, *rar1* mutants do not display any obvious developmental defects (Muskett *et al.*, 2002). *RAR1* is absent from the genome of *Chlamydomonas*, which conspicuously also lacks NB-LRR proteins (Merchant *et al.*, 2007).

Thirdly, SGT1 is known to function closely with RAR1 and HSP90, although its role is ambiguous (Azevedo *et al*., 2002). While SGT1 is implicated in some NB-LRR-associated immune responses, it is also associated with non-NB-LRR immune sensors as well as growth and development in *Arabidopsis.* A requirement for *SGT1* in *Nicotiana benthamiana* was illuminated using virus-induced gene silencing (Gilbert & Wolpert, 2013). *SGT1* is a highly conserved eukaryotic protein which has a general role in disease resistance, interacting with co-chaperones RAR1 and HSP90 (Azevedo *et al*., 2006; Peart *et al.*, 2002). In *Nicotiana benthamiana,* silencing *SGT1* resulted in decreased LOV1 protein concentration, suggesting that *SGT1* is involved in maintaining LOV1 (NB-LRR) stability.

A chaperone protein is likely required for *SSN* NB-LRR stability. Because we did not observe developmental deficits in second-site *Arabidopsis* mutants, RAR1 may be the primary suspect in identifying the gene of interest. Known functions of SGT1 suggest that loss-of-function mutations of this chaperone complex proteins may result in developmental deficits. RAR1's known functions are NB-LRR stability (Peart *et* al., 2002). Therefore, we could plausibly identify RAR1 as the chaperone protein required for *SSN* function.

Our second aim was to evaluate the relationship between rust resistance and victorin sensitivity in oats to establish if the *Vb* and *Pc2* genes share identity. As with *Arabidopsis*, EMS mutagenesis was used to induce point mutations. M1 plants were generated from 168 pools of M0 plants (encompassing approximately 2000 individuals) and screened for resistance to Victoria Blight and insensitivity to victorin. From this population, nine mutants displaying loss-of-function in the *Vb* gene were identified. The victorin insensitive phenotype was confirmed in the M2 generation and subsequently screened rust resistance. Seven of the nine mutants displayed susceptibility to *P. coronata*. Interestingly, two mutants showed a different response to *P. coronata*. Mutant 4-1 displayed weak susceptibility to crown rust while mutant 21-1 was resistant to the disease.

These data strongly suggest that the *Vb/Pc-2* genes are the same. In seven of nine mutants, loss of victorin sensitivity also conferred a loss of resistance to crown rust. This is consistent with previous studies that have genetically linked victorin sensitivity and the rust resistance response, and demonstrated that the victorin response resembles that of immune defense (Wolpert *et al.,* 2002). Presuming the *Vb/Pc*-2 genes do share identity,

there are two plausible mutagenic outcomes producing a rust-resistant, victorin-insensitive mutant as revealed in mutant 21-1. First, a second-site mutation could give rise to a rust-resistant mutant. A well substantiated model for the victorin-triggered response in *Arabidopsis* involves the NB-LRR, LOV1, the product of a dominant "victorin-sensitivity gene" as well as TRXh5 which binds to victorin. A protein (perhaps a thioredoxin, as in *Arabidopsis*) which interacts with victorin and AvrPc2 may be guarded by the *Vb/Pc2*-encoded NB-LRR. The interaction of the two effectors with the guardee likely differ. A mutation in the guardee could affect the interaction such that the AvrPc2 effector is recognized while victorin is not, thus, yielding resistance to both *P. coronata* and *C. victoriae*.

Another plausible explanation is that the *Vb/Pc-2* gene possesses a partial loss-of-function mutation. Distinct from a loss-of-function mutation, partial loss-of-function yields an altered phenotype. In the case of mutant 21-1, a partial loss-of-function mutation yields a presumed NB-LRR which would interact with the AvrPc2 effector either directly or associated with its target to confer resistance to *P. coronata*. However, the altered NB-LRR would not recognize victorin via TRXh5, yielding a mutant which displays both victorin insensitivity and rust resistance. The weak phenotype of mutant 4-1 may also conform to these explanations. A similar partial loss-of function mutation in a *Vb/Pc2*-encoded NB-LRR or its guardee may result in a weak interaction with AvrPc2 to trigger attenuated immune signaling downstream. In this way, an attenuated NB-LRR activation may display slight susceptibility to *P. coronata*. Another alternative explanation exists. The NB-LRR that presumptively mediates the rust and victorin responses may have been altered in such a way that it cannot initiate cell death, which is a

critical part of the victorin sensitivity response (Curtis & Wolpert, 2002), but may not be required for rust resistance. Other immune implications related to *P. coronata* resistance may remain unaffected. In rare cases, mutagenesis may have produced this particular partial loss-of-function.

With respect to oats, a mutagenic analysis was conducted to examine the hypothesis that the *Vb* and *Pc2* genes share identity. Our results, in conjunction with the results of other investigations, support the assertion that the *Vb/Pc-2* genes are indeed the same. We also identified interesting phenotypes resulting from likely NB-LRR partial loss-of-function mutations. In *Arabidopsis*, we identified a functional requirement for a gene other than the *SSN* gene. This gene could plausibly encode the chaperone protein *RAR1,* which has been implicated in NB-LRR stability and is unlikely to yield mortality in its absence.

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