Specific Missense Alleles of the Arabidopsis Jasmonic Acid Co-Receptor COI1 Regulate Innate Immune Receptor Accumulation and Function

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Abstract

Plants utilize proteins containing nucleotide binding site (NB) and leucine-rich repeat (LRR) domains as intracellular innate immune receptors to recognize pathogens and initiate defense responses. Since mis-activation of defense responses can lead to tissue damage and even developmental arrest, proper regulation of NB–LRR protein signaling is critical. RAR1, SGT1, and HSP90 act as regulatory chaperones of pre-activation NB–LRR steady-state proteins. We extended our analysis of mutants derived from a rar1 suppressor screen and present two allelic rar1 suppressor (rsp) mutations of Arabidopsis COI1. Like all other coi1 mutations, coi1r²p missense mutations impair Jasmonic Acid (JA) signaling resulting in JA–insensitivity. However, unlike previously identified coi1 alleles, both coi1r²p alleles lack a male sterile phenotype. The coi1r²p mutants express two sets of disease resistance phenotypes. The first, also observed in coi1-1 null allele, includes enhanced basal defense against the virulent bacterial pathogen Pto DC3000 and enhanced effector-triggered immunity (ETI) mediated by the NB–LRR RPM1 protein in both rar1 and wild-type backgrounds. These enhanced disease resistance phenotypes depend on the JA signaling function of COI1. Additionally, the coi1r²p mutants showed a unique inability to properly regulate RPM1 accumulation and HR, exhibited increased RPM1 levels in rar1, and weakened RPM1-mediated HR in RAR1. Importantly, there was no change in the steady-state levels or HR function of RPM1 in coi1-1. These results suggest that the coi1r²p proteins regulate NB–LRR protein accumulation independent of JA signaling. Based on the phenotypic similarities and genetic interactions among coi1r²p, sgt1b, and hsp90.2r²p mutants, our data suggest that COI1 affects NB–LRR accumulation via two NB–LRR co-chaperones, SGT1b and HSP90. Together, our data demonstrate a role for COI1 in disease resistance independent of JA signaling and provide a molecular link between the JA and NB–LRR signaling pathways.


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Introduction

During their life cycle, plants have to fend off microbial pathogens including fungi, bacteria, viruses, and nematodes. To protect themselves, plants rely on the innate immune system of each plant cell to detect pathogen attack and subsequently activate disease resistance responses. The plant immune system relies on two inter-related branches. The first branch utilizes pattern recognition receptors (PRRs) to identify conserved pathogen associated molecular patterns (PAMPs). This recognition then initiates PAMP-triggered immunity (PTI) [1–3]. Although PTI can restrict further colonization in some cases, successful pathogens are still able to evade or suppress PTI with their effectors [4]. These proteins contribute to pathogen virulence by interfering with various plant defense-related cellular processes. However, effectors can also be recognized by the intracellular NB–LRR receptor proteins of the plant innate immune system [5]. Recognition of effectors results in effector-triggered immunity (ETI) and is the second branch of the plant immune system [1–3]. NB–LRR proteins contain a centrally located nucleotide binding site (NB) domain and a C-terminal leucine-rich repeat (LRR) domain. Mammalian NB–LRR containing (NLR) proteins mediate analogous processes in mammalian innate immunity [6]. NB–LRR-mediated ETI is typically associated with a form of programmed cell death at the infection site termed the hypersensitive response (HR) [1–3]. If not controlled, this strong response...
Author Summary

To detect pathogen attack and subsequently trigger defense responses, plants utilize immune receptors composed of a nucleotide binding site (NB) domain and a C-terminal leucine-rich repeat (LRR) domain that function inside the cell. To identify regulators of NB–LRR protein accumulation and activity, we performed a genetic screen in the model plant Arabidopsis thaliana to isolate mutants that affect NB–LRR protein accumulation levels and NB–LRR triggered disease resistance. Here, we introduce two mutant alleles of COI1, a gene which encodes a well-characterized receptor for the phytohormone Jasmonic Acid (JA). It is widely accepted that COI1 is involved in JA signaling-dependent disease resistance. However, our new coi1 mutants affected NB–LRR accumulation in a manner independent of the JA signaling pathway. This indicated that not all disease resistance effects of COI1 require JA signaling. We also observed a link between COI1 and the RAR1-SGT1b-HSP90 co-chaperone complex, which plays a critical role in regulation of NB–LRR protein accumulations.

can lead to unnecessary tissue damage. Proper regulation of HR and therefore appropriate regulation of pre-activation, resting state NB–LRR proteins is critical [7–9]. Genetic analyses uncovered three genes, RAR1, SGT1 and HSP90, as key regulators of NB–LRR stability and activity [10–18]. RAR1, SGT1 and HSP90 proteins can interact independently with one another [13,14,16], and can cooperate as a molecular chaperone complex to regulate NB–LRR stability and function. HSP90 is usually thought to be the central subunit of the complex [19,20]. RAR1 affects the conformational dynamics of HSP90, and modulates the “lid-open” conformation required for loading client NB–LRR proteins [21,22]. However, the functional mechanism by which the RAR1-SGT1b-HSP90 complex maintains NB–LRR levels remains poorly understood.

As highly conserved proteins, SGT1 and HSP90 also interact with each other in mammalian cells, and play essential roles in mammalian immune responses mediated by NLR proteins. By co-immunoprecipitation experiments, both SGT1 and HSP90 were found to associate with many NLR proteins including NOD1 (Nucleotide-binding Oligomerization Domain 1), NOD2 (Nucleotide-binding Oligomerization Domain 2), and NALP3 (NACHT, LRR and PYD domains-containing Protein 3) [23,24]. In mammalian cells, treatment with geldanamycin (GDA), a chemical inhibitor of HSP90, impaired NOD2-induced NF-κB activity and NALP3-mediated inflammatory responses [24]. Knockdown of HSP90 by RNAi or GDA treatment also reduced the accumulation levels of NOD1 and NOD2 [23]. These results demonstrated that mammalian HSP90 is required for both NLR stability and function. In contrast, mammalian SGT1 is only required for NLR functions such as NOD1-mediated cytokine production, NOD1-mediated cell death, and NALP3-mediated inflammatory responses, but not for NLR stability [23,24]. Plant SGT1, however, functions in both NB–LRR activity and stability [25]. Moreover, mammalian SGT1 knockdown reduced the association between HSP90 and the NALP3 LRR domain, indicating that mammalian SGT1 functions as a co-chaperone of mammalian HSP90 to regulate client NLR protein [24]. Unlike plant RAR1, CHP1 (CHORD-containing Protein 1), a homolog of RAR1 in mammals, is not involved in regulating NLR protein accumulation or function [24]. Taken together, the SGT1-HSP90 chaperone complex has functions for mammalian NLR protein stability and activity, analogous to its functions for plant NB–LRR biology [19,20].

During infection, both host plants and pathogens regulate phytohormone signaling to enhance their defense and virulence respectively. Jasmonic Acid [JA] controls a well characterized example of phytohormone signaling required for both disease resistance and effector-induced susceptibility that is an outcome of the suppression of PTI [26,27]. The JA receptor, COI1, is the key regulator of JA signaling [28–31]. Mutations in COI1 cause defects in JA responses and reproductive development [32,33]. Of note, mutations in COI1 also affect, negatively or positively, disease resistance against various plant pathogens [29,33–41].

COI1 encodes an F-box protein that is a component of the SCF<sup>COI1</sup> (Skp1/Cullin/F-box<sup>COI1</sup>) E3 ubiquitin ligase complex [31,32,42]. The function of COI1 is to specifically bind target proteins to promote ubiquitination and degradation by the 26S proteasome [31]. It is therefore assumed that COI1 regulates JA signaling and disease resistance via degradation of specific proteins. The connection between JA signaling and SCF<sup>COI1</sup>-mediated protein degradation has been confirmed. The JASMONATE ZIM DOMAIN (JAZ) family proteins act as repressors of MYC2, a key transcriptional activator of JA responses, by directly interacting with MYC2, JA-ile, a bioactive JA conjugate, induces the degradation of JAZ proteins by enhancing the protein interaction between JAZs and COI1, and thus de-represses JA-related transcription activation [28,29,31,43]. The JAZ and MYC proteins also play a role in disease resistance. Overexpression of JAZ1A3A, a C-terminal deletion form of JAZ1, led to enhanced disease resistance against <i>Pto</i> DC3000 in Arabidopsis [29]. The triple mutant for transcription factor genes MYC2, MYC3, and MYC4, which are all repressed by JAZ proteins, was as resistant against <i>Pto</i> DC3000 as the <i>coi1</i> mutant [44].

In this study, we extend our previously described suppressor screen for new mutants that recover impaired <i>Rps5</i> function in <i>rar1</i> [21]. We introduce two novel missense alleles of COI1 that suppress the disease resistance phenotypes associated with <i.rar1</i> mutation. Surprisingly, these two <i>coi1 rar1 suppressor</i> (<i>sps</i>) alleles are completely fertility, in contrast to the male sterility associated with all other <i>coi1</i> mutant alleles [32,43,45]. Like <i>sgr1</i> and the <i>hsp90.2</i><sup>hap</sup> alleles [21], these two <i>coi1</i><sup>sps</sup> alleles interact with <i.rar1</i> to restore the disease resistance responses mediated by some NB–LRRs and the accumulation of at least RPM1. Moreover, we demonstrate that overexpression of <i>Sgt1b</i> can partially inhibit the <i>coi1</i><sup>sps</sup>-enhanced accumulation of RPM1 and <i>Rpm1</i>-mediated disease resistance in <i.rar1</i>. We also observe non-allelic non-complementation, a rare genetic interaction, between <i>coi1</i><sup>sps</sup> mutants and <i>hsp90.2</i><sup>hap</sup> mutant. These results support the hypothesis that <i>coi1</i><sup>sps</sup> proteins regulate NB–LRR levels via SGT1b and HSP90.

Results

Identification of new alleles of COI1 and of the <i>rsp3</i> mutant

To identify new genes that act with <i>RAR1</i> to regulate NB–LRR accumulation and activation, we performed a suppressor screen for new mutants which can suppress the disease susceptibility observed in <i.rar1-21</i> (a stop mutation in Q12) [21]. Five <i.rar1 suppressor</i> (<i>sps</i>) mutants were identified from approximately 200,000 M<sub>2</sub> plants from 50 M<sub>2</sub> pools that recover resistance responses to both <i>Pto</i> DC3000(<i>avrPphB</i>) and <i>Pto</i> DC3000(<i>avrRpt</i>D) [21]. Based on map-based cloning and subsequent allele sequencing, two of the five mutants were found to have mutations in <i>COI1</i> (<i>At2g39940</i>). To follow accepted nomenclature conventions, we designated these two mutant alleles, <i>coi1-21</i><sup>sps</sup> and <i>coi1-22</i><sup>sps</sup>,...
respectively (Figure 1). Based on disease symptoms after inoculation of Pto DC3000(\textit{avrRpm1}) on backcross F1 and F2 populations, both of the \textit{coi1} mutants were completely recessive (Table S1). This conclusion was also confirmed by growth assays of Pto DC3000(\textit{avrRpm1}) in backcross F1 plants (Figure 2). The \textit{coi1-21} mutation is a G/A transition which leads to a G330E missense change in the COI1 protein. The \textit{coi1-22} mutation is a G/A transition resulting in a G434E missense change in the protein. Both mutations are within conserved LRR domains (Figure 1).

Using the crystal structure of the Arabidopsis COI1 protein, we observed that neither \textit{coi1mut} mutation is localized in the interfaces of COI1 that make up the ASK1-binding region and the ligand-binding pocket [31].

In addition, another \textit{rar1} suppressor (\textit{rsp}) mutant called \textit{rsp3} was isolated from this screen. \textit{rsp3} suppressed all known \textit{rar1} phenotypes, and was localized in a 7 Mbp region on chromosome I (Figure S1). A single allele, dominant mutation was identified in \textit{rsp3}; its detailed characterization is beyond the scope of this work.

**Figure 1. Mutations identified in COI1.** The F-box domain and the LRR domain are shown in dark and light gray, respectively. The allele designation and associated amino acid change is shown in relation to its linear position. New alleles introduced in this paper are shown with larger font. doi:10.1371/journal.pgen.1003018.g001

**Figure 2. COI1 and HSP90 interact genetically to regulate disease resistance.** Bacteria Pto DC3000(\textit{avrRpm1}) were hand-infiltrated into leaves of each indicated genotype and counted at day 0 and day 3. Error bars represent 2 \times SE. The result displayed is one of two independent analyses giving similar results. doi:10.1371/journal.pgen.1003018.g002
COI1 and HSP90 interact genetically to regulate disease resistance

The disease resistance restoration phenotypes of hsp90.2-7r and either coi1rr alleles in rar1 are fully recessive with respect to their respective wild type phenotypes [21], Figure 2. We monitored in planta growth of Pto DC3000 (avrRpm1) to measure RPM1-mediated disease resistance in F1 plants of hsp90.2-7r × coi1rr crosses (Figure 2). The resulting F1 plants were as resistant to Pto DC3000 (avrRpm1) as their parental coi1rr plants. We also tested F1 plants of crosses between hsp90.2-7r and either coi1rr allele for disease symptoms after inoculation of Pto DC3000 (avrRpm1). The F1 plants displayed resistance against Pto DC3000 (avrRpm1) as well as disease resistance responses. HR mediated by many NB–LRR proteins.

RAR1

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RAR1

whether they have any NB–LRR specificity in their suppression of disease susceptibility phenotype in players of crosses between hsp90.2-7r and coi1-16. The resulting F1 plants were as resistant to Pto DC3000 (avrRpm1) and Pto DC3000 (EV) in a rar1 background (Figure 3A, 3B). The increase in disease resistance against Pto DC3000 (EV) was even higher than that caused by the coi1rr alleles (Figure 3B). To our surprise, coi1-16 resulted in the recovery of RPM1-myc accumulation in rar1, but coi1-1 did not (Figure 3C). However, coi1-16 and coi1-1 express equivalent enhanced disease resistance in rar1. Thus, the “restoration” of disease resistance responses against Pto DC3000 (avrRpm1) that we observed in coi1-1 rar1 is not due to restoration of NB–LRR protein levels, but rather to bypass suppression of rar1 disease susceptibility. This is likely caused by enhanced basal defense possibly related to the antagonistic relationship between JA- and SA-dependent signaling (Figure 3).

RAR1

The growth of Pto DC3000 (avrRpm1) and Pto DC3000 (EV) at 3 dpi was about the same in coi1-16 rar1 plants (Figure 3). Thus, the restored disease resistance in coi1-16 rar1 is likely due to enhanced basal defense, not RPM1 function, although there is a restoration of RPM1-myc accumulation in coi1-16 rar1.

Since the coi1-1 null allele cannot suppress rar1, we suggest that the coi1rr alleles and coi1-16 are recessive gain-of-function alleles for the rar1 suppression phenotypes. They are also loss-of-function alleles for the JA response phenotypes as detailed below.

The coi1rr mutations negatively regulate RPM1-dependent HR in otherwise wild-type plants

We introduced the coi1rr alleles into an isogenic RAR1 background using marker-assisted breeding (see Methods). To further study the role of COI1 in regulating RPM1 function, we inoculated both coi1rr alleles, coi1-1 and coi1-16 plants with Pto DC3000 (avrRpm1) and measured bacterial growth (Figure 4B). The coi1rr and coi1-16 mutants were as resistant as wild type. The coi1-16 mutant displayed slightly enhanced resistance compared with wild type. We also measured RPM1-mediated HR in these coi1 single mutants using the ion leakage assay (Figure 4C). Surprisingly, both coi1rr alleles weakly suppressed RPM1-mediated HR. We crossed RPM1-myc into these coi1rr, coi1-1 and coi1-16 single mutants and measured RPM1-myc protein levels (Figure 3C). We observed no obvious changes in RPM1-myc levels in any of the single coi1 mutants. We conclude from these data that coi1rr mutations differentially regulate RPM1 function in rar1 or RAR1 backgrounds.

Increased RPM1 accumulation in coi1rr and coi1-16 is post-transcriptionally regulated

Loss of COI1 leads to elevated levels of salicylic acid (SA) in plants [37], and elevated SA levels can induce the expression of some NB–LRR-encoding genes [51–53]. NB–LRR expression is not changed in rar1 (Figure S4, [49]). We measured RPM1 mRNA levels in the coi1rr, coi1-1, and coi1-16 mutant plants in the context of wild-type RAR1 by RT-qPCR in order to determine whether the increased RPM1-myc protein levels noted in coi1rr and coi1-16 were due to enhanced transcription. Wild type and rar1 plants were used as controls. We detected no enhancement of RPM1
inhibition of seedling growth [32,43,45]. We compared JA-insensitivity phenotypes of the coi1<sup>iso</sup> alleles to coi1-1 using a growth inhibition assay where plants were grown in the presence of MeJA, a functional JA derivative (Figure 5). Like coi1-1, the MeJA-treated coi1<sup>iso</sup> seedlings grew on MeJA-containing media, while the growth of wild type seedlings was severely inhibited (Figure 5A). MeJA treated coi1<sup>iso</sup> seedlings were clearly smaller than the untreated seedlings, suggesting that the coi1<sup>iso</sup> alleles are not as insensitive to JA as coi1-1. We quantified these phenotypes with a root elongation assay (Figure 5B). The null allele coi1-1 displayed root growth inhibition of only about 14% in the presence of 50 μM MeJA. Compared with coi1-1, coi1-16 and both coi1<sup>iso</sup> alleles displayed intermediate insensitivity to MeJA treatment. Their root growth was inhibited about 27%, 30% and 42% respectively, while the root growth inhibition was more than 60% in wild type seedlings. Thus, the coi1<sup>iso</sup> alleles are JA-insensitive.

JA signaling is important in disease resistance responses. coi1 and other JA insensitive mutants exhibit enhanced resistance to the virulent bacterial strain Pto DC3000(EV) [29,37,38]. We measured the growth of Pto DC3000(EV) in our coi1<sup>iso</sup> alleles, coi1-1, and coi1-16 (Figure 4A). The coi1<sup>iso</sup> alleles also displayed enhanced resistance to Pto DC3000(EV), although the increase in the coi1<sup>iso</sup> alleles was slightly lower than in the reference alleles coi1-1 and coi1-16.

The coi1<sup>iso</sup> alleles are not null alleles

The coi1<sup>iso</sup> alleles are quantitatively different than the coi1-1 null allele with respect to JA responses (Figure 5B) and enhanced resistance to Pto DC3000(EV) (Figure 4A). We noted decreased COI1 protein accumulation levels in coi1-21<sup>iso</sup>, coi1-22<sup>iso</sup> and coi1-16 plants compared to wild type and rar1 plants (Figure 3C). As expected, no detectable amount of COI1 protein was observed in coi1-1. The residual accumulations of COI1 protein confirmed that the coi1<sup>iso</sup> alleles and coi1-16 are not COI1 null alleles.

The sgt1b mutant is insensitive to JA responses

To determine whether other NB–LRR regulators function in regulating JA responses, we tested the JA response in the mutants of three NB–LRR co-chaperones, RARI, SGT1b and HSP90.2 by the root elongation assay (Figure S3). All rar1 and hsp90.2 mutants were as sensitive to MeJA treatment as wild type, suggesting that neither RARI nor HSP90.2, plays a role in JA responses. As expected, the sgt1b mutant displayed an obvious insensitivity to MeJA [54]. We also noted MeJA insensitivity in the rar1 sgt1b double mutant (Figure S3). These results suggest that SGT1b is the only member of RAR1-SGT1-HSP90 NB–LRR co-chaperone complex required for JA signaling.

COI1 mutations do not affect the levels of RARI, SGT1b, or HSP90 accumulation

coi1 mutations restored the disease resistance responses mediated by three NB–LRR proteins in rar1 (Figure 3A, Figure S2A and S2B) and thus possibly suppressed rar1 via effects upon NB–LRR regulators that control the accumulation, and hence the function, of multiple NB–LRR proteins. To examine this possibility, we determined the accumulation levels of three NB–LRR regulators, RARI (Figure S2A), SGT1b (Figure S2B), and HSP90 (Figure S2C), in the coi1<sup>iso</sup>, coi1-1 and coi1-16 mutants in either RARI or rar1 backgrounds. These coi1 mutants did not exhibit any dramatic change of RARI, SGT1b or HSP90 protein levels. Therefore, the coi1<sup>iso</sup> and coi1-16 alleles do not suppress rar1 influencing by regulating the steady state levels of RARI, SGT1b and/or HSP90.

The coi1<sup>iso</sup> alleles are JA–insensitive

COI1 has an essential role in JA signaling; all previously isolated COI1 mutations caused insensitivity to JA-mediated

mRNA levels among the tested coi1 mutants (Figure S4), indicating that the coi1<sup>iso</sup> and coi1-16 alleles restore RPM1 protein levels by a post-transcriptional mechanism in rar1.

The coi1<sup>iso</sup> alleles are JA–insensitive

COI1 has an essential role in JA signaling; all previously isolated COI1 mutations caused insensitivity to JA-mediated
SGT1b antagonizes coi1rsp-mediated RPM1 accumulation and RPM1-dependent disease resistance in rar1

The coi1rsp mutants displayed opposite phenotypes: increased NB–LRR accumulation and function in rar1 and decreased NB–LRR HR function in RAR1. A similar combination of phenotypes was previously observed in sgt1b as an rar1 suppressor [25]. The sgt1b mutation enhanced RPS5 accumulation and consequent restoration of RPS5-mediated disease resistance in rar1, but did not restore RPS5-triggered HR in RAR1 [25]. This similarity implies that coi1rsp mutants might regulate NB–LRR proteins by inhibiting the function of SGT1b and hence mimic sgt1b phenotypes.

Based on this hypothesis, we expected that a high dose of SGT1b would attenuate the rar1 suppression phenotypes of the coi1rsp alleles. To test this, we introduced a 35S:SGT1b-HA construct into coi1-21rsp rar1 plants containing RPM1-myc. Compared with parental coi1-21rsp rar1 plants, four independent T3 lines that expressed relatively high levels of SGT1b::HA exhibited both reduced RPM1-myc levels (Figure 6A) and RPM1-mediated disease resistance (Figure 6B). However, the RPM1 accumulation and RPM1-mediated disease resistance observed in these T3 plants were still much higher than rar1 plants (Figure 6A, 6B). These results demonstrated that modest over-expression of SGT1b can partially inhibit the rar1 suppression phenotypes of coi1rsp alleles. As a control, we measured the growth of Pto DC3000(EV) in the plants used in the Pto DC3000(avrRpm1) growth assay. No enhanced growth of Pto DC3000(EV) was observed in these T3 lines (Figure 6C), demonstrating that the reduction of RPM1-mediated disease resistance in 35S:SGT1b-HA transgenic plants are not due to a decrease in basal defense.

In addition, we measured the HSP90 protein levels and RPM1-myc mRNA levels in the transgenic plants used in the western blot analysis. No obvious decrease of HSP90 protein level (Figure S6A) or RPM1-myc mRNA level was detected (Figure S6B), indicating that the reductions of RPM1-myc accumulation in 35S:SGT1b-HA transgenic plants are not due to the decrease of HSP90 accumulation or the silencing of RPM1-myc gene.

Discussion

We initially performed a suppressor screen for mutants that could recover the diminished NB–LRR RPS5-mediated disease resistance phenotype of rar1 [21]. These suppressors were isolated in the null rar1-21 background (Figure S5A), and thus likely represent mutations that either bypass or counteract rar1. We reported two novel HSP90 alleles derived from this screen that function to mimic the effects of RAR1 on the HSP90 lid open/close cycle required to stabilize NB–LRR clients [21]. Here, we detail the characterization of two coi1 alleles, coi1-21 and coi1-22 also identified in this screen (Figure 1), and we note that a third single allele locus defined by rsp3 has characteristics that suggest it might encode another new player in the regulation of NB–LRR accumulation (Figure S1). Because rsp3 is a single, dominant allele, its description beyond the mutant phenotype was not pursued as part of this study.

The F-box protein COI1 is a core component of the receptor complex for jasmonate [JA] [28,29,31]. In plants, mutations in COI1 impair all known JA responses and thus result in insensitivity to JA or functional JA derivatives [32,33,37,43,45]. As expected,
insensitivity and intact fertility of the coi1R alleles are likely due to lower accumulation of functional COI1R proteins in these mutants. In other words, the coi1R mutations, G330E and G434E, cause relatively weaker impairments of the COI1 protein stability and activity than the other reported coi1R missense alleles.

**COI1 functions in both basal defense and ETI**

Mutations in COI1 affect, negatively or positively, disease resistance against various plant pathogens [29,33–41]. It is widely accepted that the defense phenotypes of coi1 depend on signaling antagonism between SA and JA signaling pathways [33]. COI1 mutations disable JA-signaling and consequently enhance SA signaling and SA-induced defense responses by an as yet unknown mechanism.

In Arabidopsis, resistance against the virulent hemi-biotrophic pathogen Pto DC3000 is a measure of basal defense [56]. In our study, all four tested coi1 alleles, coi1-21R, coi1-22R, coi1-1, and coi1-16 displayed enhanced disease resistance against Pto DC3000(EV) in both var1 and RAR1 backgrounds (Figure 3B, Figure 4A). These results correspond to previously published data [29,37,38], and confirm that COI1 represses basal defense, likely via JA-Sa antagonism. Besides enhanced basal defense, the coi1 alleles also displayed enhanced ETI against Pto DC3000(avrRpm1) (Figure 3A, Figure 4B). Hence, COI1 also inhibits ETI. Since the enhancement of ETI was found in var1 mutant plants, RAR1, which is necessary for NB-LRR-mediated ETI in this and many other cases, is not required by COI1 to repress ETI.

**A plausible mechanism explaining COI1 effects on NB-LRR accumulation in rar1 and RAR1**

Although all four coi1 alleles we analyzed restored resistance against Pto DC3000(avrRpm1) in var1 (Figure 3A), we could classify them into three classes based on how they influence RPM1 accumulation and RPM1-mediated immune response (Figure 3C, Figure 4C). Class I, represented by the null allele coi1-1, does not alter RPM1 levels. Class II, represented by coi1-16, enhances RPM1 levels in var1 and has no effect on RPM1-mediated HR in RAR1. Class III, represented by coi1-21R and coi1-22R, enhance RPM1 levels in var1, but reduce RPM1-mediated HR in RAR1. Since the null coi1-1 does not exhibit any detectable effect on RPM1 accumulation, the enhancement of RPM1 levels in var1 is a gain-of-function phenotype conferred by the COI1 mutant proteins accumulating in coi1-16 and the two coi1R alleles. However, these alleles are all recessive for JA response phenotypes. The coexistence of these distinct genetic characteristics demonstrates that coi1-16 and coi1R alleles are recessive gain-of-function alleles which have lost the JA signaling function of COI1, but gained new function, likely via interfering with the activity of other protein(s). RPM1 is associated with, and activated at, the plasma membrane; there is no current evidence suggesting that it shuttles into the nucleus [50,57]. COI1 is expected to be localized in the nucleus, because it binds to the nucleus-localized JAZ proteins [58]. A biochemical mechanism to explain our genetic results would require a reconciliation of these findings. There may be sufficient coi1R protein at the plasma membrane to mediate the effects on RPM1 that we describe. Further, our inference that COI1 has a wild type function in mediating NB-LRR protein accumulation is consistent with suggestions that nucleo-cytoplasmic shuttling is required for the function of at least a subset of NB-LRR proteins [3].

Some publications suggest that the “target” protein with which recessive gain-of-function alleles interfere can share functional redundancy with it [14,59–62]. We found that mutants of two NB-LRR co-chaperones, SGT1b and HSP90, have phenotypic similarities with coi1R alleles [14, 21, 25]. These include (Table S5):

![Figure 5. coi1R alleles are insensitive to JA.](image-url)

(A) Seedlings of the indicated genotypes were grown on MS medium (control) or medium containing 10 or 50 μM MeJA. (B) Inhibition of root elongation by 50 μM MeJA in at least twenty seedlings of indicated genotypes. This assay was performed independently three times with similar results. doi:10.1371/journal.pgen.1003018.g005
Figure 6. SGT1b over-expression antagonizes coi1<sup>−/−</sup>-dependent RPM1 accumulation and RPM1-mediated disease resistance in rar1. (A) Western blot analysis of RPM1-myc and SGT1b-HA protein levels in indicated genotypes. RuBiSco levels stained by Ponceau S serve as loading control. The result displayed is one of three independent blots giving similar results. (B–C) Bacterial growth assays were performed independently three times (Pto DC3000<sup>(avrRpm1)</sup>) and twice (Pto DC3000<sup>(EV)</sup>) with similar results. 

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1) enhanced NB–LRR accumulation in rar1: RPM1 in hsp90.2<sup>−/−</sup> rar1 [21], RPS5 in sgt1 rar1 [25], and RPM1 in coi1<sup>−/−</sup> rar1 (this work); 2) impaired NB–LRR-mediated HR in RAR1: RPM1-mediated HR in hsp90<sup>−/−</sup> [14], RPS3-mediated HR in sgt1b [29], and RPM1-mediated HR in coi1<sup>−/−</sup> (this work). COI1 is an F-box protein which is a component of an SCF complex. Both SGT1b and HSP90 have been reported to associate and function with various SCF complexes in plants [13,54,63,64]. These findings collectively imply that SGT1b and/or HSP90 are candidate target proteins of coi1<sup>−/−</sup> proteins in suppressing rar1.

Since the coi1<sup>−/−</sup> alleles did not affect steady state SGT1b levels (Figure S5B), coi1<sup>−/−</sup> alleles might inhibit SGT1b activity to suppress the rar1 phenotype of reduced NB–LRR accumulation. To test this hypothesis, we overexpressed SGT1b in a coi1-21<sup>−/−</sup> rar1 background. The rar1 suppression phenotypes of coi1-21<sup>−/−</sup>, restored RPM1-myc accumulation and RPM1-mediated disease resistance, were partially complemented by SGT1b overexpression (Figure 6A, 6B). This result supports our hypothesis, and suggests that SGT1 functions with COI1 to regulate NB–LRR accumulation. On the other hand, the incomplete complementation could mean that we need higher levels of SGT1b over-expression, or that coi1<sup>−/−</sup> proteins also down-regulate the activity of other targets, such as HSP90. Our speculation is supported by the non-allelic non-complementation observed between coi1<sup>−/−</sup> mutants and hsp90.2<sup>−/−</sup> mutant (Figure 2, Table S2). This specific genetic relationship suggests that COI1 and HSP90 physically interact with each other or belong to the same protein complex.

The RAR1-SGT1-HSP90 chaperone complex has been related to the SCF complex by two sorts of evidence: 1) SGT1b and HSP90 associate and function with various SCF complexes [13,54,63,64]. RAR1 associates with the COP9 signalosome (CSN) which can inactivate the SCF complex [13,64,65]; 2) The SCF<sup>CPR1</sup> complex negatively regulates the pre-activation steady state stability of two NB–LRR proteins, SNC1 and RPS2, via the COP9 signalosome (CSN) which can inactivate the SCF complex [13,64,65]; 2) The SCF component SKP1 is required for NB–LRR N protein-mediated resistance response against tobacco mosaic virus (TMV) [64]. This relationship suggests that RAR1-SGT1-HSP90 chaperone complexes function with an SCF-mediated protein degradation pathway to control the accumulation levels of NB–LRR protein and thus avoid inappropriate NB–LRR activation [19]. The phenotypes observed in our recessive gain-of-function coi1<sup>−/−</sup> mutants support this hypothesis. The coi1<sup>−/−</sup> mutants suppressed the rar1 mutant for reduced NB–LRR RPM1 accumulation, and showed non-allelic non-complementation with hsp90.2<sup>−/−</sup> mutant. Moreover, overexpression of SGT1b partially inhibited the phenotypes of the coi1<sup>−/−</sup> mutants. Similar to sgt1b and hsp90.2<sup>−/−</sup> mutants, coi1<sup>−/−</sup> mutants caused impaired HR function when moved to a wild type background. The sum of these results is consistent the idea that the F-box protein COI1 functions with RAR1-SGT1-HSP90 chaperone complex and consequently affects NB–LRR protein accumulation and function.

Materials and Methods

Plant lines

We used coi1-1 [32] and coi1-16 [45] as reference alleles. For the pathology analyses and root elongation analyses, mutant lines used (all in Col-0 background) were rar1-21 [18], rpm1-1 [67], rps3-2 [68], rps2-101c [69], sgt1<sup>1-1</sup> [17], rar1-21 sgt1<sup>1-1</sup> [25], hsp90.2-2 [14], hsp90.2-5 [14], hsp90.2-7 [21] and hsp90.2-8 [21].
Pathogen strains, inoculation, growth quantification, and ion leakage assay

Pro DC3000 derivatives containing pVSP61(EV), avrRpm1, avrPphB, and avrRpt2 were maintained as described [71]. Plant inoculations and bacterial growth assays were performed as described (spray-inoculation [21]; dip-inoculation [72]; hand-inoculation [25]). The HR test and ion leakage assays were carried out as described [21].

Hyaloperonospora arabidopsidis (Hpa) isolate Emw1 was used to inoculated ten-day-old cotyledons of plants as described [21]. Asexual sporangiophores were counted 7 days post-inoculation on at least 30 cotyledons for each genotype.

Identification and map-based cloning of mutations in COI1

The ral suppressor screen was previously described [21]. Standard genetic analyses and map-based cloning were performed as described [21]. We used 892 disease resistant F2 individuals to define a 60 Kb interval on the chromosome II containing COI1. By sequencing COI1 in the originally isolated ral suppressor mutant, a G/A transition at position 1849 (nucleotide positions relative to the translation start site of the published sequence of COI1; ATG39940) was identified in col1-2198. The other mutant, col1-2299, also contains a G/A mutation at position 2161 in COI1. To obtain col1-2198 and col1-2299 single mutants, we backcrossed the col1-98 alleles into an isogenic RAR1 background. PCR-based dCAP markers were designed for selecting these two col198 mutants.

MeJA treatment

For growth inhibition assays, seedlings were grown on MS medium with different concentrations of Methyl Jasmonate (MeJA) under 24 h light for 4 d. Then seedlings were transferred to new MS medium with or without 50 μM MeJA, and grown for additional 4 d. Root elongations during these four days were measured.

Western blots

For detection of RPM1-myc in the genotypes mentioned in this study, we introduced by crossing and segregation the mutants into plants expressing RPM1-myc from the native RPM1 promoter as described [14]. The protein extraction and western blot were performed as described [14]. For detection of SGT1b-HA in plants, the protein extraction and western blot were carried out based on the protocol that was previously used for RPS5-HA [25]. The anti-COII1 antiserum was kindly provided by Xiaoxin He (Tsinghua University, Beijing, China). The protein extraction and western blot were performed as described [42], anti-SGT1 and anti-RAR1 polyclonal antibodies against the full length SGT1b and full length RAR1 with C-terminus GST tag were generated in rabbits (custom products of Cocalico Biologicals, Inc.). anti-HSP90-2 was the product of Agrisera company (Swedish). The detailed protocols for detection of SGT1a, SGT1b, RAR1, and HSP90 proteins are provided as Text S1.

RT-qPCR

Plant RNA was extracted with RNeasy Plant Mini Kit (Qiagen). To eliminate DNA contamination, RNA was purified by Turbo DNA Free Kit (Ambion) and RNeasy Mini Kit (Qiagen). 2 μg RNA was reverse transcribed with Random Decamers and RETROscript kit (Ambion). RT-qPCR was performed in a total volume of 25 μl (12.5 μl SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μl cDNA, 1 μl Primer 1 (10 μM), 1 μl Primer 2 (10 μM) and 10 μl H2O) with MJ White 96-well plate and a DNA Engine OPTICON 2 system (MJ Research). A negative control was included as a no template control. The reaction was run at 95°C for 5 min, followed by 40 cycles at 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. Dissociation analysis was performed after each reaction to confirm the specificity. The relative expression of RPM1/RPM1-myc gene in different genotypes was calculated by 2ddCt method (User Bulletin #2, Manual of Applied Biosystems). The primers were newly designed or obtained from previous publication [73], and are provided as Text S1.

Supporting Information

Figure S1 rps3 can suppress all known ral mutant phenotypes. In addition to two hsp90.29 and two col198 mutants, we isolated a fifth mutant, named rps3 (ral suppressor 3), from the ral suppressor screen. Based on disease symptoms after inoculation with Pro DC3000(avrRpm1), we determined that rps3 is dominant. The results of bacterial growth experiments showed that the rps3 mutant suppressed ral for disease resistance functions of RPS5 (Figure S1A), RPM1 (Figure S1B) and RPS2 (Figure S1C). rps3 also fully suppressed the decreased basal disease resistance phenotype of ral (Figure S1D). In addition to disease resistance, RPM1-mediated HR in ral was also suppressed by rps3 (Figure S1E). We also noted that rps3 partially suppressed the loss of RPP4 function in ral following infection with Hpa Emw1 (Figure S1F). Finally, we also found that rps3 mutant suppressed the lowered accumulation of RPM1 in ral (Figure S1G). The combined phenotypes of rps3 ral mutant demonstrated that rps3 suppressed, fully or partially, all known ral phenotypes. Using map-based cloning, we localized rps3 mutation in a 7 Mb (from 15.9 Mb to 22.9 Mb) mapping interval on chromosome I (Figure S1H). No NB-LRR regulator has been found in this interval. However, because it is a single dominant allele, further characterization of rps3 is beyond the scope of this paper. (A) Bacterial growth assays of (A) Pro DC3000(avrPphB), (B) Pro DC3000(avrRpm1), (C) Pro DC3000(avrPphB) and (D) Pro DC3000(EV). Leaves of each indicated genotype were dip-inoculated [1]. Bacteria were counted at day 0 and day 3. Error bars represent 2× SE; (E) Conductivity measurements after inoculation with high concentration of Pro DC3000(avrRpm1) (5×107 cfu/ml). Error bars represent 2× SE; (F) 10-day-old cotyledons were inoculated with Hpa isolate Emw1. Asexual sporangiophores were quantified 7 days after inoculation on cotyledons for each of the indicated genotypes [2]. (Fp: sporangiophore); (G) Western blot analysis of RPM1-myc protein levels in indicated genotypes. RuBiCo levels stained by Ponceau S serve as loading control; (H) The positions of the Simple Sequence Length Polymorphisms (SSLP) markers used for rough map-based cloning on chromosome I. rps3


mutation was localized in the mapping interval between marker T2K12 (15.9 Mb) and F19K23 (22.9 Mb). The pathogen growth and HR assays were performed independently a minimum of three times with similar results. The RPM1-myc blot displayed is one of three independent blots giving similar results.

Figure S2 coi1ΔΔ alleles suppress some, but not all ral1 phenotypes for NB-LRR function. (A-B) Bacterial growth analysis of Pto DC3000 (avrPsbB) (A) and Pto DC3000 (avrRpt2) (B). Leaves of indicated genotypes were dip-inoculated [1]. Bacteria were counted for day 0 and day 3. Error bars represent 2× SE. Pairwise comparisons for all means for bacterial growth on day 3 were performed with One-Way ANOVA test followed by Tukey-Kramer HSD at 95% confidence limits; (C) Conductivity measurements after inoculation with high concentration Pto DC3000 (avrRpm1) (5×10^7 cfu/ml). Error bars represent 2× SE; (D) 10-day-old cotyledons were inoculated with Hpa isolate Ernval. Asexual spargiophores were quantified 7 days after inoculation on cotyledons for each of the indicated genotypes [2]. (Sp. spargiophore). The pathogen growth and HR assays were performed independently a minimum of three times with similar results.

Figure S3 coi1ΔΔ and sgt1b are insensitive to McJA. Inhibition of root elongation by 50 μM McJA for the indicated genotypes. The root elongation assay was performed three times with similar results. At least fifteen seedlings per genotype were measured in each repeat. Error bar represents 2×SE.

Figure S4 coi1ΔΔ and coi1-16 mutations do not enhance RPM1 transcript levels. RT-qPCR analysis of the expression of RPM1 for indicated genotypes. The result displayed is one of three independent RT-qPCRs giving similar results.

Figure S5 COI1 mutants studied express wild type levels of RAR1, SGT1 and HSP90 proteins. Western blot analysis of SGT1b, SGT1a, RAR1 and HSP90 protein levels for the indicated genotypes. RuBiCo levels stained by Ponceau S serve as loading control. The western blots were performed twice independently with similar results.

References

Figure S6 The reductions of RPM1-myc levels in the 35S::SGT1b-HA transgenic plants are not due to the decrease of HSP90 protein level or the silencing of RPM1-myc gene. (A) Western blot analysis of RPM1-myc, SGT1b, SGT1a, and HSP90 protein levels for the indicated genotypes. RuBiCo levels stained by Coomassie Brilliant Blue serve as loading control; (B) RT-qPCR analysis of the expression of RPM1 and RPM1-myc for the indicated genotypes. The western blot and RT-qPCR assay were performed independently a minimum of two times with similar results.

Table S1 Both of the coi1ΔΔ mutants are completely recessive.

Table S2 Non-allelic non-complementation between coi1ΔΔ and hsp90.2ΔΔ mutants. RPM1-mediated resistance was tested by spray-inoculation with Pto DC3000 (avrRpm1). Disease symptoms were evaluated 5 days after inoculation.

Table S3 Phenotypic similarities among coi1, sgt1b, and hsp90.2 mutants.

Text S1 Supporting information including the primers and corresponding enzymes for selecting of mutations, the primers for making 35S::SGT1b-HA construct, the primers used for RT-qPCR, and additional information of Western blots.

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Author Contributions
Conceptualized and designed the experiments: YH E-HC DAH PT JLD. Performed the experiments: YH E-HC PT. Analyzed the data: YH E-HC DAH JLD. Contributed reagents/materials/analysis tools: YH E-HC DAH PT. Wrote the paper: YH E-HC JLD.

Specific Alleles of coi1 Suppress ral1