An Allele of Arabidopsis COI1 with Hypo- and Hypermorphic Phenotypes in Plant Growth, Defence and Fertility

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Abstract

Resistance to biotrophic pathogens is largely dependent on the hormone salicylic acid (SA) while jasmonic acid (JA) regulates resistance against necrotrophs. JA negatively regulates SA and is, in itself, negatively regulated by SA. A key component of the JA signal transduction pathway is its receptor, the COI1 gene. Mutations in this gene can affect all the JA phenotypes, whereas mutations in other genes, either in JA signal transduction or in JA biosynthesis, lack this general effect. To identify components of the part of the resistance against biotrophs independent of SA, a mutagenised population of NahG plants (severely depleted of SA) was screened for suppression of susceptibility. The screen resulted in the identification of intragenic and extragenic suppressors, and the results presented here correspond to the characterization of one extragenic suppressor, coi1-40. coi1-40 is quite different from previously described coi1 alleles, and it represents a strategy for enhancing resistance to biotrophs with low levels of SA, likely suppressing NahG by increasing the perception to the remaining SA. The phenotypes of coi1-40 lead us to speculate about a modular function for COI1, since we have recovered a mutation in COI1 which has a number of JA-related phenotypes reduced while others are equal to or above wild type levels.

Introduction

The ability of plants to prevent pathogen colonization relies on a complex network of genes and phytohormones. Salicylic acid (SA) is a well known hormone essential for activating plant basal defence responses, particularly against biotrophic pathogens (reviewed by [1]). An imbalance in basal levels of SA can dramatically alter plant resistance. For instance, Arabidopsis thaliana (Arabidopsis) plants with high levels of SA are more resistant to pathogens such as the bacteria Pseudomonas syringae pv. tomato isolate DC3000 (Pto) [2], while plants with lower SA levels are less resistant to Pto and other pathogens [3]. Furthermore, it has been shown that transgenic plants expressing the salicylate hydroxylase gene from Pseudomonas putida (NahG) can rapidly degrade SA [3] and are therefore more susceptible to biotrophic pathogens [4] such as Pto [5]. Once activated, SA resistance triggers a number of defence or pathogenesis-related genes including PR1. This gene is widely used as a marker for biotic stress and is required for various types of resistance, including Systemic Acquired Resistance (SAR). SAR acts to protect systemic leaves following earlier localized pathogen inoculation [6]. Considering the array of resistance responses to biotrophs, there is evidence for part of the resistance response being independent of SA [7].

A phytohormone with an intricate relationship with SA is jasmonic acid (JA). JA is required for a wide range of plant functions, from pollen maturation to activating defence responses against necrotrophic pathogens (reviewed by [8]). It also plays a minor role in activating defence responses against biotrophs, since exogenous application of jasmonates, i.e. methyl ester jasmonate (MeJA), can trigger defence against Pto, a response that appears dependent on activation of NPR1 [9]. JA is also important for inducing systemic induced susceptibility (SIS, [10]), which, in contrast to SAR, induces susceptibility in systemic leaves. As with SA, there are several genes that play a significant role in JA signal transduction, but only its receptor, COI1, is absolutely required for inducing all related phenotypes. SA and JA have been shown to negatively regulate each other, although there are examples of synergistic effects [reviewed in [11]].

This study aims to investigate resistance responses that are independent of SA. Although there are no viable biosynthetic mutants that are completely deficient of SA [12], NahG plants are severely depleted in SA [3]. Using a mutagenised NahG population and screening for suppressors of NahG susceptibility, we aimed to...
identify and characterize parts of the resistance response that would normally be masked by the abundance of SA. The results presented here show that part of the SA-independent defence response is dependent on JA perception. In addition, we show that an allele of COI1 displays a number of JA-related phenotypes reduced while others are equal to or above wild type levels.

Results

Design and implementation of a NahG suppressor screen

In a previous screen for loss of resistance to Pto, one of us (P.T.) recovered a promising Arabidopsis mutant, lra5 [13]. Further characterization showed that lra5 was in fact a stray NahG plant that contaminated the screen (data not shown). The NahGCW line was found to have some islands in the genome from the accession Ws-0 (data not shown), hence the name. Then, it was backcrossed five times with the accession Col-0. The transgene was inserted between the genes At2g46970 and At2g46980, and there were no differences in susceptibility to Pto with other Col-0 or Ws-0 NahG lines (data not shown).

We took advantage of the detailed characterization of this line to elucidate how the SA-dependent and independent branches of the resistance response interact, by using NahGCW to screen for mutations that suppress the susceptibility to Pto. Before embarking on the NahGCW suppressor screen, the conditions were optimized using several Arabidopsis mutants with an enhanced resistance against Pto [1]. We generated double mutants between NahGCW and cpr5 [14], epr5 [15], ddx1 [16], and lsd1 [17]. With these plants, we fine-tuned a medium throughput screening protocol that would detect suppressors of NahG susceptibility. Figure S1 shows the proof of concept after optimizing the inoculations. With two inoculations of Pto, wild-type plants of the ecotypes Col-0, Ws-0 and Lao-0 can overcome the pathogen and grow almost unaffected, while NahGCW plants died or were severely affected. The enhanced disease resistance mutants in combination with NahGCW produced a small but detectable suppression of NahG, but in the case of epr5 NahGCW, there is a strong suppression of the susceptibility (Figure S1, also described by [15]).

From 60 independent M2 families, 89 candidates were recovered and 40 selected for further characterization. These 40 putative mutants were crossed with Col-0, and their F2 progeny were inoculated with Pto. We identified 12 intragenic and 28 extragenic suppressors. Three of the intragenic suppressors were selected and characterized further to confirm that they were allelic and less susceptible to Pto than the parental line NahG (Figure S2A and S2B, respectively).

An extragenic suppressor, coi1-40, was selected for further characterization. In our conditions coi1-40 was not different to wild type in all the gross morphological phenotype (data not shown). This mutant was shown to contain a single nuclear mutation, which was recessive and effectively suppressed the susceptibility of NahG. coi1-40 was mapped to Chromosome II, between the markers C2-12916335 and BLO2-18012804 (an interval of 5.1 Mb).

Response to pathogens of coi1-40

Since NahG plants accumulate a very low but discernable level of SA [18], it was important to ascertain if the suppression of susceptibility in coi1-40 plants was related to alterations in SA levels or other mechanisms. The steady state levels of SA in coi1-40 were similar to Col-0, and coi1-40 NahGCW accumulated similar levels compared to NahGCW (Figure S3).

Identification of the suppressor mutants was based on visual inspection of disease symptoms. It is possible, however, that the reduced macroscopic disease symptoms did not reflect reduced pathogen growth. Therefore, more accurate measurements of Pto growth were performed. These measurements confirmed that coi1-40 is able to suppress the growth of Pto in a NahG background (Figure 1A). In fact, the single mutant was more resistant than Col-0, even in the NahG background, demonstrating that coi1-40 has a heightened basal resistance. The status of resistance can also be evaluated by the levels of the protein PR1 [19]. Upon Pto inoculation PR1 was strongly induced in coi1-40 compared to the Col-0 control, however, in coi1-40 NahGCW no induction was evident (Figure 1B). The same membranes were probed with anti-Rubisco as an internal control (Figure 1B).

NahG is not only susceptible to compatible pathogens like Pto, but also to some incompatible and non-host pathogens [20]. Figure 1C-D displays the behaviour of coi1-40 when inoculated with Pto(avrRpm1) [21] or Pto(avrRpt2) [22]. The presence of the avrRpm1 or avrRpt2 effector converts Pto into an incompatible pathogen in the presence of the resistance genes RPM1 [23] and RPS2 [24], respectively, and coi1-40 did not suppress the susceptibility to either effector (Figure 1C and D). Analogous results were obtained when the genotypes were inoculated with the non-host pathogens Pseudomonas phaseolicola (Pph) and Pseudomonas tabaci (Ptab) (Figure 1E-F).

As mentioned previously, pathogen resistance can be activated independently of SA signalling. To assess the resistance of coi1-40 against necrotrophic pathogens, which is dependent on JA signalling [25], the mutants were inoculated with Plectosphaerella cucumerina (Figure 2), coi1-40 showed a marked increase in susceptibility to P. cucumerina one week after inoculation. Figure 2B shows the leaves of an experiment when the sampling is done two weeks after inoculation; note that coi1-40 NahGCW was slightly less susceptible than coi1-40 alone. cpr5, a mutant more resistant to P. cucumerina, is included as a control [26]. OCP3 is a homeodomain transcription factor, and a mutation in this gene renders plants more resistant to necrotrophic pathogens without affecting the resistance to biotrophs [26].

JA defence-related phenotypes of coi1-40

Given the enhanced susceptibility of coi1-40 to P. cucumerina and that resistance against necrotrophs is dependent on JA, we reasoned that it would be interesting to test a series of JA related phenotypes in coi1-40. Figure 3A shows the resistance induced by MeJA when Pto is inoculated one day later [27]. Both Col-0 and NahGCW respond to MeJA with a small but reproducible reduction in the levels of Pto, compared to the negative control jin1 (JasmonateInsensitive 1, [28]). In these conditions, no resistance response was evident for coi1-40 and coi1-40 NahGCW in response to MeJA. SAR has been shown to be dependent on JA, [29] and in accordance wild-type Col-0 plants displayed SAR. However, NahGCW and jin1 (Figure 3B), coi1-40 and coi1-40 NahGCW had no SAR and Pto grew better in SAR conditions, especially for coi1-40 plants. This phenotype has been called SIS [10], and it is a systemic effect of coronatine. Some isolates like Pto are able to produce this chemical, a molecular mimic of the iso-leucine conjugate of jasmonic acid [JA-Ile, [30]], that functions as a virulence factor [31]. We inoculated coi1-40 and coi1-40 NahGCW and their controls with a Pto strain that lacks coronatine (Pto(fa2), [31]), and a wild-type Pto strain (Figure 3C). Pto(fa2) had a reduced growth in wild-type plants, compared to Pto, while in coi1-40 and coi1-40 NahGCW there were no detectable differences, or the differences were opposite to those on wild-type plants (Figure 3C).

One of the hallmarks of JA signalling in defence is the negative crosstalk with SA signal transduction [32]. Therefore, we measured the perception of benzothiadiazole (BTH, an analogue
of SA, [33]) to check the status of crosstalk in coi1-40 plants. Wild-type, jin1 and coi1-40 plants had a considerable reduction in fresh weight, when compared to npr1 and wild type plants (Figure 3D). The increase in sensitivity to BTH for these mutants is consistent with less negative crosstalk from JA to SA signalling pathways.

JA growth-related phenotypes of coi1-40

To determine whether perception of JA in coi1-40 was affected during development, we studied the phenotypes of coi1-40 alongside coi1-1. The receptor of JA, COI1 (Coronatine Insensitive 1, [34]), is an F-box protein and it is a key regulator of JA signalling, therefore null alleles are insensitive to JA. The plants were grown in vitro in the presence and absence of MeJA. In the absence of MeJA, all plants were comparable, while in the...
The number of trichomes is another developmental phenotype partially dependent on JA [30]. We found that the number of trichomes for coi1-40 was enhanced in the presence of MeJA (Figure 5D), and that the morphology was strikingly different to wild-type plants (Figure 5E, S6).

**coi1-40 is an allele of coi1**

coi1-40 and coi1-1 displayed quite different JA-dependent phenotypes, such as coi1-40 being fertile. However, both mutants shared certain phenotypes such as dry weight of the root system in MeJA, and coi1-40 mapped to an interval that contains COI1 (At2g39940). In order to check for complementation, the F1 plants from a cross between coi1-1 and coi1-40 or Col-0 and coi1-40 were obtained and grown in MeJA-containing media (Figure 4D). The F1 from the coi1-1 by coi1-40 cross showed identical phenotypes to coi1-40, when root length, number of lateral roots, and number of trichomes were assessed. This suggests that coi1-40 is an allele of COI1 (Figure S7) or that both mutations are in different genes that interact genetically and could give rise to non-allelic, non-complementation. However, in the F2 all plants were resistant to MeJA, and 3 in every 4 were similar to coi1-40, while 1 in every 4 was similar to coi1-1 (data not shown). Therefore, both mutations are allelic, and coi1-40 has allele-specific phenotypes. The F1 plants from the cross between Col-0 and coi1-40 displayed a wild type phenotype when grown in the presence of MeJA. (Figure 4D) and the F2 segregated 3:1 (Col-0 to coi1-40), indicating that coi1-40 is a recessive mutation. Sequencing of COI1 in coi1-40 revealed a single canonical EMS mutation in the gene, changing residue 22 from glutamic acid to lysine, indicating again that coi1-40 is allelic to coi1-1 (Figure S5).

The lateral root, length of the main root, and trichome phenotypes were dominant in coi1-40 with respect to coi1-1, but recessive with respect to wild type COI1 (Figure 4D and Figure S7). Once the mutation was identified, 60 F2 from coi1-1 × coi1-40 and 60 F2 from Col-0 × coi1-40 crosses were analyzed with a molecular marker and the lateral root phenotype was found to cosegregate with the coi1-40 marker in both populations (data not shown). The trichome phenotype was also visually observed as cosegregating with the molecular marker in 70 F2s from the cross coi1-1 × coi1-40, due to the low level of trichomes in coi1-1 (Figure 5D and E).

**JA-induced expression of COI1-dependent genes in coi1-40**

The differences in some of the JA-dependent phenotypes shown by Col-0 and the coi1-1 and coi1-40 mutants should mirror a molecular footprint. Therefore, we analyzed the effect of MeJA on the expression of six important genes involved in JA-related phenotypes (Figure 6). The *ASA1* gene (Anthranilate Synthase 1, [35]) may modulate auxin biosynthesis in response to JA thus regulating lateral root formation. We analyzed the expression of this gene to address whether the lateral root phenotype of coi1-40 was dependent of this node (Figure 6A). No significant differences were observed; hence the phenotype of coi1-40 might be independent of this auxin pathway. The expression of *COI1* was also examined and found to be identical in the two coi1 alleles (Figure 6B). *PAP1* and *PAP2* (Production of Anthocyanin Pigment 1 and 2, [39]) are two *MYB* genes that mediate the JA-dependent anthocyanin biosynthesis. It has been reported that the induction of both genes by JA is also dependent of COI1 [37]. In response to MeJA, coi1-40 maintained *PAP1* (Figure 6C) and displayed reduced levels of *PAP2* (Figure 6D). However, both displayed higher expression levels than in coi1-1. The expression of two further genes was also assessed, *MYC2* and *VSP1*. *MYC2* is a
central transcription factor for most JA-induced responses and
VSP1 (Vegetative Storage Protein 1, [40]) is a specific marker induced
by JA. In both we found a stronger difference between these
alleles. There is a certain amount of JA signalling that goes
through coi1-40 inducing the expression of MYC2 (Figure 6E) and
VSP1 (Figure 6F) to considerable levels, which could explain some
of the phenotype differences between coi1-1 and coi1-40.

We complemented the coi1-40 mutation by crossing it with a
transgenic line that express COI1-Flag under the control of the
promoter 35S [41]. The coi1-40 35S:COI1-Flag behaved as the
control coi1-1 35S:COI1-Flag homozygous line for relative length of
the root (Figure 7A and B) and number of trichomes (Figure 7C
and 7D) demonstrating that these phenotypes are caused by the
mutation in coi1-40.

Discussion

A NahG extragenic suppressor

Resistance against biotrophs in plants depends largely on SA
accumulation [42]. As NahG plants have reduced levels of SA, this
provides a background where mutations that enhance the
resistance in an SA independent manner are easily recognizable.
Here we report the screening of a NahG mutagenised line, which
resulted in the identification of intragenic (mutations in the NahG
transgene itself), and extragenic mutations. An extragenic mutant,
coi1-40, was fully characterized, and shown to be more resistant
against biotrophic pathogens than the parental lines both in the
presence or absence of NahG (Figure 1A).

coi1-40 is an interesting and informative allele by itself, as
described below. However, our initial objective was to analyze the
interaction between SA-dependent and independent branches of
the resistance response. coi1-40 was found in our screen because it
suppresses the susceptibility of NahG (Figure 1A). This suppression
is produced by two mechanisms. First, the insensibility of the
mutant to coronatine depletes the advantage that this chemical
gives to the pathogen [31]. Second, since the steady-state level of
PR1 protein in coi1-40 is almost undetectable but is strongly
induced upon Pto inoculation, (Figure 1B), we speculate that coi1-
40 increases the sensitivity to pathogen signals that trigger
resistance. As coi1-40 was identified as an allele of COI1, mutating
coi1 would potentially increase sensitivity to SA, since in wild type
plants there is negative crosstalk between JA and SA [43]. The
same increased sensitivity is shown with respect to BTH
(Figure 3D).

Therefore, one of the mechanisms of coi1-40 suppressing NahG
susceptibility is SA-independent (coronatine is no longer a
virulence factor), but the other mechanism is SA-dependent
(enhanced perception of SA). The two incompatible (Figure 1C

Figure 3. Pathogen resistance phenotypes of coi1-40 related to JA. coi1-40 and its controls were tested for: (A) Methyl jasmonate (MeJA)
induced resistance. 17-day-old-plants were treated with either 100 μM MeJA (with 0.1% DMSO and 0.02% Silwet L-77) or a mock solution. One day
later, Pto was inoculated and its growth measured as in Figure 1A. (B) Systemic Acquired Resistance. Three leaves of 28-day-old plants were hand
infiltrated with either Pto(avrRpm1) or a mock solution. Two days later, Pto was inoculated and its growth in systemic leaves measured as described
(see Methods). (C) Coronatine as a virulence factor. Bacteria with coronatine (Pto, COR+) or without coronatine (Pto(cfa2), COR-) were inoculated and
their growth measured as in Figure 1A. (D) Negative crosstalk with SA. Plants of the indicated genotypes were treated four times with either 350 μM
benzothiadiazole (BTH) or a mock solution, and their weight was recorded when 21 days old. The ratio between BTH treated and mock treated is
shown as a percentage, with npr1 included as control. jin1 is included in all the panels as a control of no response to JA. Asterisks indicate statistically
significant differences from the mock treatment (P<0.05 one asterisks, P<0.01 two) using the Student’s t-test (two-tails).
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and 1D) and the two non-host interactions (Figure 1E and 1F) were not affected by coi1-40. Therefore, increased sensitivity to SA and lack of coronatine recognition conferred by coi1-40 mutants has no effect on these interactions although it showed enhanced basal resistance. On the other hand, the response to P. cucumerina in coi1-40 was severely compromised (Figure 2) pointing to an impairment in the JA-disease resistance against necrotrophic pathogens. Interestingly, the response to P. cucumerina also shows the negative regulation from SA to JA. Thus, when the leaves were sampled two weeks after inoculation (Figure 2B), coi1-40 NahG were slightly less susceptible than coi1-40, likely due to the reduced perception of SA in NahG, that leads to the increased perception of JA by a weak allele of COI1.

Figure 4. Response of coi1-40 to JA in vitro. coi1-40 and its controls were tested for: (A) Phenotype in plates. The indicated genotypes were grown in plates with Johnson’s Media [67] supplemented with 50 μM MeJA. The pictures were taken 20 days after germination with the same settings and in the same experiment. In plates without MeJA the plants were the same size (data not shown). (B) Length of primary root. The plants were grown as described in (A), with and without 50 μM MeJA. At 10 days old, the lengths of the roots were measured in both conditions, and their ratio (MeJA treated divided by mock treated) expressed as a percentage. (C) Lateral roots. The plants were grown as described in (A), with and without 50 μM MeJA. At 14 days old, the number of lateral roots longer than 0.2 mm was counted in both conditions with the help of a magnifying glass. Note that in some genotypes like Col-0, the root does not grow in MeJA and therefore it is not possible to count lateral roots (marked as "n.d." in the figure). Since coi1-1 is not fertile, the number of lateral roots without MeJA was not counted (marked as not determined -n.d.- in the figure). (D) Phenotype of F1s between coi1-40 and Col-0 and between coi1-40 and coi1-1. The plants were grown as described in A, with 50 μM MeJA. The pictures were taken when the plants were 20 days old.

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coi1-40 differentiates phenotypes related to JA in roots

coi1-40 shares several phenotypes with other JA mutants such as jin1 or coi1-1. Thus, like coi1-1 [34], coi1-40 is fully susceptible to P. cucumerina (Figure 2). When exogenously applied, MeJA is able to induce a small resistance against subsequent Pto infections [27]. coi1-40 does not trigger this resistance (Figure 3A), nor SAR (Figure 3B, [44]), although it has an enhanced perception of SA and its analogues (Figure 3D, [45]).

There are certain differences between the phenotypes induced by the coi1-40 and coi1-1 alleles (Table 1). Four of these phenotypes indicate that coi1-40 is a hypomorph, i.e. intermediate between Col-0 and the coi1-1 null mutant. These unrelated phenotypes include the relative length of roots growing in MeJA plates, the senescence induced by JA, the production of carotenoids induced by JA, and the fertility. While two are produced by exogenous application of MeJA, two are responding to endogenous levels.
While two are on sterile plates, two are in soil. From the mentioned phenotypes, it is tempting to merely assign a weak or leaky character to \textit{coi1}-40, however, there are three phenotypes in which \textit{coi1}-40 behaves as a hypermorph; lateral root growth, trichome development and SIS.

The development of lateral roots is orchestrated by the distribution of auxins (basipetal in the root and acropetal in the leaf [46]). Auxin application stimulates the formation of lateral roots [47], while inhibitors of auxins prevent the formation of them [46]. Therefore, the increased number of lateral roots in \textit{coi1}-40 may be brought about by an increase in production or perception of auxins. The gene \textit{ASA1} (\textit{ANTHRANILATE SYNTHASE} \textit{a}1) is an auxin biosynthesis gene responsible for lateral root formation in the presence of JA [35]. Expression analysis has shown that the expression of \textit{ASA1} is \textit{COI1} dependent (Figure 6A, [35]). Therefore, \textit{ASA1} could act to integrate JA and auxin signalling. While the line of argument for auxins being involved in the formation of lateral roots in \textit{coi1}-40 is appealing, this could not be verified experimentally. Thus, \textit{ASA1} was not induced by MeJA in \textit{coi1}-40 (Figure 6A), nor was there any apparent phenotype of the \textit{coi1}-40 mutant in 2,4-D plates (data not shown).

\textit{coi1}-40 produces more trichomes in response to JA than the wild type

\textit{Arabidopsis} responds to wounding or MeJA applications by increasing the number of trichomes in the newly formed leaves [48]. Surprisingly, not all mutants in JA signalling are defective in trichome response to JA [38]. In \textit{coi1}-40 this response to JA is hypermorphic (Figure 5D and E). The production of trichomes in \textit{Arabidopsis} involves a complex genetic model, including \textit{Glabra3} (\textit{GL3}; [49]), among other genes. JA induces the expression of \textit{GL3}, setting in motion the formation of trichomes [38]. In \textit{coi1}-40, the levels of \textit{GL3} are not altered upon MeJA treatment (data not shown).

\textit{SIS}

\textit{Pto} grows better in \textit{coi1}-40 plants where SAR has been triggered, which could indicate that, like other mutants in JA signalling, \textit{coi1}-
40 is negatively affected in SAR and displays a hypermorphic SIS [10]. Although the initial observation of SIS was obtained from incubation with a virulent strain, there is evidence that an avirulent strain can also trigger SIS [10]. While in a wild type plant this effect would be overcome by SAR, *coi1*-40 allows separating these two opposing tendencies, favouring SIS (Figure 3B).

**Figure 6. Allele specific molecular phenotypes of coi1-40.** Col-0, coi1-1, and coi1-40 plants were grown both in mock and 50 μM MeJA plates. RNA was extracted 10 days after germination, and transcript levels for the following genes were measured by RT-qPCR: (A) ASA1; (B) COI1; (C) PAP1; (D) PAP2; (E) MYC2; (F) VSP1. The levels of expression are normalized to three reference genes and to the level of Col-0 in mock. doi:10.1371/journal.pone.0055115.g006

**Behaviour of F1s**

The lateral root and trichome phenotypes are dominant with respect to coi1-1; one copy of coi1-40 increases both the number of lateral roots and trichomes if the other allele is coi1-1 (Figure 4D and Figure S7). This fact implies that any explanation of the mentioned phenotypes by secondary EMS mutations in *NahGCW* is highly unlikely, since all the phenotypes are dependent on the locus COI1. In addition, these phenotypes cosegregate perfectly in...
a dominant fashion with a molecular marker for coi1-40 in an F2 segregating family of coi1-1 x coi1-40. Similarly, in the cross coi1-40 x Col-0, the lateral root phenotype cosegregates perfectly in a recessive fashion with the same marker. In contrast, the lateral root phenotype was not seen in F2 populations from NahG x Col-0, or NahG x Laer-0, (data not shown).

**coi1-40 and other coi alleles**

coi1-40 shares certain phenotypes with coi1-20 [50]. This allele is also resistant to Pto, and induces PR1 strongly upon inoculation. However, coi1-20 is male sterile, and the double coi1-20 NahG does not suppress NahG susceptibility [50]. Other alleles like coi1-15 and 18 are also male sterile. The mutations in coi1-15 and 18 are frameshifts that introduce stop codons [51] while the mutation in coi1-20 is unknown. coi1-16 is fertile at temperatures below 20°C; however, root growth inhibition and JA-responsive promoter activity are not restored at lower temperatures [52]. Recently, coi1-16 was used to recover loci that suppress the ABA signalling pathway, since coi1-16 is also hypersensitive to ABA in seed germination [53]. The molecular lesion in coi1-16 results in a change of leucine to phenylalanine in the leucine-rich repeat. Lately, two more alleles, coi1-21 and coi1-22 were described as
Table 1. Summary of the phenotypes that differentiate the coi1-40 and coi1-1 alleles.

| Genotypes     | Col-0 | coi1-40 | coi1-40×coi1-1 F1 | coi1-1 |
|---------------|-------|---------|------------------|--------|
| Root length†  | ++    | +++     | +                | +++    |
| Senescence‡   | +++   | +++     | n.d.             | +      |
| Carotenoids†  | +++   | +       | n.d.             | +      |
| Fertility     | +++   | +++     | +                | 0      |
| Lateral roots†| +     | +++     | +++              | +      |
| Trichomes†    | ++    | +++     | +++              | +      |
| SIS           | +     | ++      | n.d.             | 0 [10] |

†Measured in plants grown on MeJA supplemented plates.
‡Senescence induced by MeJA.

A modular model for COI1 function

We propose two models to explain the disparity of phenotypes for coi1-40. The first one implies that it is a weak allele that retains some function. The difference in phenotypes would be a question of thresholds; some phenotypes are fully functional with the level of some function. The difference in phenotypes would be a question of the modular behaviour of coi1-40, a theory that is discussed below.

Plant Growth and Inoculation

Arabidopsis thaliana was sown and grown as described [45]. Plants were grown in controlled environment rooms (CER) with days of 8 h at 21°C, 150 μmol m⁻² s⁻¹ and nights of 16 h at 19°C. For long day experiments, plants were also grown in a CER with the same conditions, except with 16 h of light and 8 h of darkness. The following genotypes were used: npr1 [58], ndr1 [59], sid2 [60], cpr5 [14], cpr5 [15], dnd1 [16], lsd1 [17], rpm1 [23], rps2 [24], nho1 [61], npk3 [26], coi1-1 [34], and jin1 [28]. The treatments, inoculations, and sampling started 30 minutes after the initiation of the artificial day to ensure reproducibility. Pseudomonas syringae pv. tomato DC3000 (Pto) was maintained as described [62]. Pto was used with the pVSP61 plasmid containing avrRpm1 [62], avrRpt2 [22], or an empty vector. Pto (cfa −) was obtained from Dr. Jeff Dangl (UNC, Chapel Hill, NC, USA). The bacteria were grown, inoculated and measured as described [63]. Systemic Acquired Resistance was performed as reported by [64], inoculating leaves with both incompatible and compatible pathogens using a blunt syringe. Pseudomonas syringae pv. phaseolicola isolate NPS3121, and Pseudomonas syringae pv. tabaci were obtained from Dr. Jeff Dangl (UNC, Chapel Hill, NC, USA). The bacteria were grown, inoculated and measured as described [63]. The experiments, at least three independent treatments were performed three independent sets of plants sown and treated on different dates.

Chemical Treatments

Benzothiadiazole (BTH, CGA 245704), in the form of a commercial product (Bion® 50 WG, a gift from Syngenta Agro S.A., Spain) was prepared in water for each treatment and applied with a household sprayer. The BTH treatments were done as described in [43] and [66]. Briefly, plants were treated with either mock or 350 μM BTH four times during two weeks, starting when the plants were one week old. Then, the fresh weight of each genotype was recorded in both treatments and expressed as percentage of fresh weight of mock-treated plants.

JA-related phenotypes

For in vitro culture, plants were grown in Johnson’s media [67] with 1 mM KH₂PO₄. When indicated, the plates were supple-
mented with either 10 or 50 μM MeJA (Duchefa, Haarlem, The Netherlands), depending on the experiment. The length of the roots was measured with ImageJ software (MIH, Bethesda, MD, USA), and the number of lateral roots, with the help of a magnifying glass. Only lateral roots longer than 0.2 mm were counted. When measuring the effect of MeJA on Pt0 growth, MeJA was applied by spray at 100 μM in 0.1% DMDO (SIGMA, St. Louis, MO, USA) and 0.02% Silwet L-77 (Crompton Europe Ltd, Evesham, UK) one day before Pt0 inoculation [60]. Senescence induced by MeJA was measured as described by [36]. For carotenoid measurements, the protocol described by [69] was followed. In order to quantify the amount of seeds produced per plant, eight coi1-40 and eight wild type plants were selected by molecular marker analysis from an F2 backcross with Col-0. Eight coi1-1 plants were also selected from a F2 population segregating for this mutation. Plants were grown in long day conditions, and when the first fruit had matured, the aerial part was covered with a paper bag to avoid loss of seeds. Once the plant had senesced, the seeds were cleaned and weighed. The number of trichomes on the fifth true leaf of 14-day-old plants grown on plates with 10 μM MeJA was determined with the aid of a magnifying glass as described by [39]. The pictures of trichomes were taken with a JSM-5410 scanning electron microscope (JEOL, Tokyo, Japan) in the Electron Microscopy Service (Universidad Politécnica de Valencia, Spain).

Western Blot

Immunodetection of PR1 protein was carried out as described [19], using an Amersham ECL Plus Western Blotting Detection Reagent (GE HealthCare, Little Chalfont, UK). The second antibody was a 1:25,000 dilution of Anti-Rabbit IgG HRP Conjugate (Promega, Madison, WI, USA). Chemiluminescent signals were detected using a LA-3000 Luminescent Image Analyzer (FujiFilm Life Science, Stamford, USA). Immunodetection of the large subunit of RuBisCO was accomplished with a LA-3000 Luminescent Image Analyzer (FujiFilm Life Science, Stamford, USA). Immunodetection of the large subunit of RuBisCO was accomplished with a LA-3000 Luminescent Image Analyzer (FujiFilm Life Science, Stamford, USA). Immunodetection of the large subunit of RuBisCO was accomplished with a LA-3000 Luminescent Image Analyzer (FujiFilm Life Science, Stamford, USA).

Mutagenesis, screening, and mapping

Once the screening conditions were established, seeds of NahG (this work), cpr1 [14], cpr5 [15], lsd1 [17], and dhi1 [16] and double mutant combinations with NahG proved by spray-inoculation with Pseudomonas syringae pv. tomato isolate DC3000 (Pto) at an OD600 of 0.1 at 28 days after germination and again one week later. The pictures were taken one week after the second inoculation. (TIF)

Figure S1 Proof of concept of the NahG suppressor screen. Three accessions (Col-0, Ws-1, and Lan-0), five single mutants (NahG, cpr1, cpr5, lsd1, and dhi1) and double mutant combinations with NahG were challenged as described in Methods. Note that with the first 40 mutants, the screen is by no means saturated. The intragenic suppressors form an internal control, since the screen has been sensitive enough to detect 12 reversions to wild type of a single locus. Assuming a Poisson distribution and the extreme scenario that all the extragenic suppressors belong to different complementation groups, the average ratio of alleles per complementation group of 1.38 implies that in the first 40 mutants there would be a maximum of 23% complementation groups not present [73]. (TIF)

Supporting Information

Table S1 Sequencing primers of coi1-40 and molecular markers.

(PDF)

RT-qPCR

Total RNA from 10-day-old plants grown on media with or without 50 μM MeJA was extracted with Trizol (Invitrogen, Barcelona, Spain), following the manufacturer’s instructions. The details of the RT-qPCR (MIQE data) are provided as Methods S1.

Figure S2 Characterization of NahG intragenic suppressors. (A) Resistance and alleleism test of intragenic suppressors. The resistance (R) of M1 intragenic suppressor plants, the non-complementation of the intragenic suppressors with Col-0, the resistance (R) evaluation of the F1 and F2, and the allele test between these suppressors was checked. For this purpose, four-week-old plants (the number indicated as ‘n’) were divided by mock treated) expressed as a percentage. The dry weight was measured in both conditions, and their ratio (MeJA treated/F2 plants appeared that were as susceptible as NahG and as resistant as Col-0, the suppressor was labelled as extragenic.

Figure S3 Salicylic acid content of coi1-40. Both free and total (free plus conjugated) Salicylic Acid is reported for 28 day-old uninoculated plants. Three samples of 100 mg leaves were frozen in liquid nitrogen. Salicylic acid measurements were performed with the biosensor AgNetochinone sp. ADPWH_lux, as described (74), (75). The SA levels of Ws-0 are similar to those in Col-0 (data not shown). (TIF)

Figure S4 Dry weight of roots growing with and without JA. The plants were grown as described in Figure 4A, with and without 50 μM MeJA. At 17 days-old, the dry weight of the roots was measured in both conditions, and their ratio (MeJA treated divided by mock treated) expressed as a percentage. The dry weight was determined after drying the roots for 48 h at 65°C. jin1 and coi1-1 mutants were used as controls. (TIF)

Figure S5 Mutation in coi1-40 and comparison of COI1 and TIR1 related F-box proteins from Arabidopsis.

Amino acid sequences of COI1, TIR1 and five other TIR1-related F-box proteins from Arabidopsis (AFB) were aligned using
CLUSTALW ([76]). Identical residues in all five AFBs and TIR1 are denoted in yellow and the substitution in the amino acid 22 responsible of the coi1-40 phenotype is denoted in red (glutamic acid (E) for lysine (K)).

**Figure S6** Details of the trichomes in two coi1 alleles.

SEM pictures of leaf epidermal trichomes of the Arabidopsis mutants coi1-1 (A and B) and coi1-40 (C and D). coi1-40 trichomes show bigger base cells, wider stem and more papillae along the trichome surface than the coi1-1 mutant (scale bar for A and C is 300 μm, scale bar for B and D is 40 μm).

**Figure S7** Analysis of the F1 between coi1-1 and coi1-40.

coi1-40, coi1-1 and its F1 were tested as described in Figure 4 and 5, for: (A) Lateral roots in plates with 10 μM MeJA. At 14 days old, the number of lateral roots longer than 0.2 mm was counted with the help of a magnifying glass. Note that there is a synergistic effect of MeJA and coi1-40 on root elongation. (B) Trichomes in plates with 10 μM MeJA. When the fifth true leaf emerged, the number of trichomes was counted with the help of a magnifying glass.

(TIF)

**Methods S1** MIQE data of the RT-qPCR presented.

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**Author Contributions**

Conceived and designed the experiments: AD BW PT. Performed the experiments: AD BW JVC PF FT. Analyzed the data: AD BW JVC PF FT. Contributed reagents/materials/analysis tools: AD BW JVC PF FT. Wrote the paper: PT.

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