Arabidopsis thaliana RESISTANCE TO FUSARIUM OXYSPORUM 2 Implicates Tyrosine-Sulfated Peptide Signaling in Susceptibility and Resistance to Root Infection

Yunping Shen, Andrew C. Diener*
Molecular, Cell and Developmental Biology, University of California Los Angeles, Los Angeles, California, United States of America

Abstract

In the plant Arabidopsis thaliana, multiple quantitative trait loci (QTLs), including RFO2, account for the strong resistance of accession Columbia-0 (Col-0) and relative susceptibility of Taynuilt-0 (Ty-0) to the vascular wilt fungus Fusarium oxysporum forma specialis matthioli. We find that RFO2 corresponds to diversity in receptor-like protein (RLP) genes. In Col-0, there is a tandem pair of RLP genes: RFO2/At1g17250 confers resistance while RLP2 does not. In Ty-0, the highly diverged RFO2 locus has one RLP gene conferring weaker resistance. While the endogenous RFO2 makes a modest contribution to resistance, transgenic RFO2 provides strong pathogen-specific resistance. The extracellular leucine-rich repeats (eLRRs) in RFO2 and RLP2 are interchangeable for resistance and remarkably similar to eLRRs in the receptor-like kinase PSY1R, which perceives tyrosine-sulfated peptide PSY1. Reduced infection in psy1r and mutants of related phytosulfokine (PSK) receptor genes PSKR1 and PSKR2 shows that tyrosine-sulfated peptide signaling promotes susceptibility. The related eLRRs in RFO2 and PSY1R are not interchangeable; and expression of the RLP nPcR, in which eLRRs in RFO2 are replaced with eLRRs in PSY1R, results in constitutive resistance. Counterintuitively, PSY1 signaling suppresses nPcR because psy1r nPcR is lethal. The fact that PSK signaling does not similarly affect nPcR argues that PSY1 signaling directly downregulates the expression of nPcR. Our results support a speculative but intriguing model to explain RFO2’s role in resistance. We propose that F. oxysporum produces an effector that inhibits the normal negative feedback regulation of PSY1R, which stabilizes PSY1 signaling and induces susceptibility. However, RFO2, acting as a decoy receptor for PSY1R, is also stabilized by the effector and instead induces host immunity. Overall, the quantitative resistance of RFO2 is reminiscent of the better-studied monogenic resistance traits.

Introduction

The fungus Fusarium oxysporum largely persists in soil as a saprophyte or in the roots of asymptomatic plants as an endophyte [1,2]. It is the rarer pathogens of F. oxysporum that are capable of invading and colonizing the vascular system of host plants, and persistence of F. oxysporum in water-conducting xylem vessels is indicative of host susceptibility [1–4]. Numerous agricultural crops, notably tomato, cotton and banana, are susceptible to debilitating vascular infection by F. oxysporum and consequently develop wilt disease [2,3,5,6].

Fusarium wilt diseases can be especially destructive to crop monocultures because pathogens are virulent in a narrow range of plant species [3,7]. In recognition of this host specificity, pathogenic isolates are classified as having special forms, or formae specialae, which typically represent one to several phylogenetic lineages in the F. oxysporum species complex [7]. Pathogens of the same forma specialis infect similar host species, and a commercial host often names the forma specialis. For instance, F. oxysporum forma specialis matthioli (FOM) is isolated from garden stock (Matthiola incana) [8].

Fusarium wilt of Arabidopsis thaliana is an ideal pathosystem for mapping, identifying and characterizing the genes responsible for host resistance to vascular wilt fungi [9]. A. thaliana is the preeminent plant for molecular genetic and genomic studies and is susceptible to infection by FOM and two other crucifer-infecting formae specialae [9,10]. In the field, F. oxysporum forma specialis conglutinans (FOC) and F. oxysporum forma specialis raphani (FOR) are recovered from diseased cabbage (Brassica species) and radish (Raphanus sativus), respectively [11]. The symptoms and progression of wilt disease in A. thaliana recapitulate the disease syndrome observed in native field hosts [8,9,12,13]. Furthermore, this experimental pathosystem preserves host specificity because A. thaliana remains completely resistant to formae specialae isolated from non-crucifer hosts [9].

Innate resistance to Fusarium wilt as well as other infectious diseases often varies among plants of the same or interbreeding species [9,14,15]. Host resistance to the infecting pathogen when available in commercially acceptable varieties and crop rotation when feasible are preferable measures to control soil-borne diseases such as Fusarium wilt because chemical treatment of fields is usually uneconomical or has too negative cost to the
The fungus Fusarium oxysporum causes debilitating vascular infections in plants and is responsible for Fusarium wilt diseases in numerous crop species. To cope with microbial pathogens such as F. oxysporum, plants express variation in resistance genes, which typically facilitate recognition of infection by pathogens and instigate a defense response. Presently, receptor-like-proteins (RLPs) are characterized as a minor class of resistance proteins with strong effect. Studying resistance to Fusarium wilt disease in the plant Arabidopsis thaliana, we discover that RFO2, a gene providing modest quantitative resistance, encodes an RLP. Extracellular leucine-rich repeats (eLRRs) of RLPs typically mediate the recognition of infection by pathogens. However, we find that the eLRRs of RFO2 do not specify resistance. The eLRRs of RFO2 and PSY1R, which is the putative receptor for an endogenous tyrosine-sulfated peptide growth regulator PSY1, share remarkable identity. Moreover, we find that PSY1 signaling promotes susceptibility to Fusarium wilt disease. From genetic analysis of a novel RLP gene that we created from both RFO2 and PSY1R, we propose a model that explains the relationship between RFO2 and PSY1R. In our model, RFO2 induces resistance because RFO2 mediates the recognition of F. oxysporum’s attempt to manipulate PSY1 signaling.

**Results**

**Mapping and identification of RFO2**

In previous genetic analysis, resistance to FOM was associated with six RFO loci in recombinant offspring of Col-0 and Ty-0 accessions of A. thaliana [9]. Plants that were heterozygous Col-0/ Ty-0 at RFO loci, including RFO2 on chromosome 1, exhibited more resistance than those that were homozygous Ty-0/Ty-0. We first confirmed the association of RFO2 with resistance by testing the resistance of progeny of a new cross, in which RFO1 and RFO2 were the only RFO QTLs segregating. RFO1 was included in the cross because previous analysis suggested that the Col-0 allele of RFO1 (RFO1-C) enhances resistance conferred by the Col-0 allele of RFO2 (RFO2-C) [9]. One parent of the cross, plant 4D2, was heterozygous at RFO2 and homozygous Ty-0 at the remaining RFO loci, including RFO1 (RFO1-T/T RFO2-C/T). The other parent was the near isogenic line 1A3, which has a small chromosomal region around RFO1-C introgressed into the Ty-0 genetic background (RFO1-C/T RFO2-T/T). Among the resulting progeny, plants that inherited RFO2-C (RFO1-C/T RFO2-C/T), which still exhibited more resistance than Ty-0 presumably because all progeny were RFO1-C/T (Figure 1A). Also, as previously observed, RFO1-C enhanced resistance conferred by RFO2-C. Both the self progeny of RFO1-T/T RFO2-C/T (4D2), which segregated for RFO2-C but lacked RFO1-C, and Ty-0, which lacked both RFO2-C and RFO1-C, exhibited similar severe symptoms (Figure 1A). Thus, RFO1-C was included in crosses to map RFO2.

Using genetic linkage analysis, we mapped RFO2 to an interval, corresponding to less than 258 kilobasepairs (kb) and fewer than 68 genes, as described in Materials and Methods, which suggested that RFO2 is the effect of a single gene.

To clone the RFO2 gene sequence, we tested whether Col-0 genomic sequence in the final RFO2 interval could enhance the resistance of RFO1-C/C RFO2-T/T (1A3). In total, 19 genomic subclones were stably introduced to line 1A3 using Agrobacterium tumefaciens-mediated transformation, and just two subclones enhanced the resistance of 1A3 (Figure S1). Independent kanamycin-resistant T1 transformants of subclone Kpn1.2 expressed more resistance to FOM than T1 transformants of subclone Avr2.1; and, similarly, T1 transformants of Sall.2 were more resistant than T1 transformants of Xba1.1 (Figure 1B).

In the two positive genomic subclones, Sall.2 and Kpn1.2, there were 13.8 kb of overlapping sequence. This overlapping environment [3,14,16]. However, genetic resistance may be poorly defined or unavailable in acceptable crop varieties. The response of wild accessions of A. thaliana to infection by FOC, FOM and FOR ranges widely from complete resistance to ready susceptibility [9]. For example, accession Col-0 exhibits complete resistance to a dose of FOM that consistently kills accession Ty-0. On the other hand, Ty-0 exhibits more resistance than Col-0 when accessions are instead infected with FOC race 1. Thus, in large part, variation in resistance is specific to the infecting forma specialis. Most researchers using the Fusarium-Arabidopsis pathosystem infect the common laboratory accession Col-0 with FOC [17–19]. Because Col-0 exhibits considerable but partial resistance to FOC, it is possible to observe either enhanced resistance or increased susceptibility in Arabidopsis mutants using the same F. oxysporum pathogen.

To improve the resistance of cultivated varieties, plant breeders exploit the genes controlling natural variation in resistance, so-called resistance genes [20]. In crosses between resistant and susceptible varieties, qualitative resistance may be inherited as multiple quantitative trait loci (QTLs) conferring polygenic resistance or as a simple discontinuous Mendelian trait conferring monogenic resistance [21]. The best-studied resistance genes confer strong monogenic resistance to specific pathogens and typically but not always code for members of the nucleotide-binding, leucine-rich repeat (NB-LRR) class of resistance proteins [22,23]. There are few examples of genes providing polygenic resistance, so it remains unclear whether particular classes of genes with common function are commonly associated with quantitative disease resistance traits [24–26].

In A. thaliana, RESISTANCE TO F. OXYSPORUM (RFO) is a polygenic trait [9]. Six RFO QTLs are detected in the recombinant progeny of Col-0 and Ty-0 accessions and account for the strong resistance of Col-0 and susceptibility of Ty-0 to FOM. RFO1, which expresses the strongest resistance among RFO QTLs, is a member of the wall-associated kinase (WAK) family of receptor-like kinase (RLK) genes. The WAK family is one of several RLK gene families whose history, genome organization and expression suggest their involvement in response to pathogens [27]. RFO1 contributes quantitatively to immunity as loss-of-function in rfo1 enhances F. oxysporum infection in the root vascular cylinder [28]. Resistance conferred by RFO2 and two other RFO QTLs appears epistatic to RFO1 and is either enhanced or dependent on the presence of RFO1 [9].

Here we show that the RFO2 QTL corresponds to diversity in receptor-like protein (RLP) genes that have conspicuous sequence similarity to the PSY1 peptide receptor gene PSY1R [29]. We find that, while the native RFO2 in Col-0 expresses modest quantitative resistance, transgenic RFO2 expresses strong, nearly qualitative resistance and confers specific resistance to FOM and no resistance to FOC. In contrast, we find that the RFO2-related PSY1R and phytosulfokine (PSK) receptor genes PSKR1 and PSKR2 promote susceptibility to F. oxysporum infection [30]. From the phenotypes and genetic interactions of chimeric RLP and RLK transgenes, we characterize the resistance function of RFO2 and propose a speculative model that connects the peptide signaling of PSY1R and pathogen-specific resistance of RFO2.
sequence was further subcloned as six restriction fragments (that are mapped in Figure 2 and Figure S1). Only 1A3 transfectants harboring constructs Hind3.1, Nsi1.2 and Nsi1.3 that include gene

At1g17250 showed enhanced resistance. Meanwhile, 1A3 transformants, harboring subclones without full-length At1g17250, namely Age1, BamH1 and Nsi1.1, were similarly affected by FOM infection as the untransformed line 1A3.

Many Sal1.2 and Kpn1.2 transgenic lines exhibited unexpectedly strong resistance as compared to the modest RFO1-C-dependent resistance expressed by RFO2 in Col-0 and Ty-0 recombinants. Analysis of a cross, in which both RFO1-C and the putative RFO2-C transgene (tRFO2) were segregating, confirmed that resistance conferred by tRFO2 was in fact independent of RFO1-C and stronger than the resistance of RFO1-C. A Kpn1.2 transgenic line (1A3×tRFO2), and Ty-0 were crossed, and the resulting F1 dihybrid RFO1-C/T, tRFO2/+/− then was backcrossed to Ty-0 to yield F1BC progeny, (i) without RFO1-C and tRFO2, (ii) with RFO1-C only, (iii) with tRFO2 only, or (iv) with both RFO1-C and tRFO2, in a ratio of 1:1:1:1 that is expected for independent assortment of RFO1 and RFO2 (Figure 1C). Plants with RFO2 were more resistant to FOM with or without RFO1-C; and, plants with tRFO2 only were substantially more resistant than plants with RFO1-C only (Figure 1C). When F1BC progeny were infected with FOC instead, plants with tRFO2 were no more resistant than plants without tRFO2 (Figure 1C). Thus, the strong resistance of tRFO2 was specific for FOM.

Because multiple RFO loci contribute to the complete resistance of Col-0, we anticipated that loss-of-function in RFO2 alone might not exhibit loss of resistance in the Col-0 genetic background. Indeed lines homozygous for T-DNA insertions in or adjacent to candidate genes in the RFO2 region were strongly resistant to FOM – see Materials and Methods for details. However, when four insertion lines were crossed to Ty-0, which halved the genetic contribution of Col-0, F1 hybrids could develop obvious wilt symptoms. The F1 hybrids of Salk_051677, in particular, were especially susceptible, and a majority of these F1 hybrids expressed symptoms, while only 20 percent of F1 hybrids of Salk_140524 were similarly affected (Figure 3A). T-DNA insertion in Salk_051677 interrupts At1g17250, the same gene that correlated with enhanced resistance in 1A3 transformants, whereas insertion in Salk_140524 interrupts At1g17200, a gene outside the overlapping sequence in Kpn1.2 and Sal1.2. Salk_051677, which was previously named Atrlp3-1 without a reported phenotype, was renamed tfo2 [31].

Although plants with genotype RFO1 tfo2 exhibited strong resistance to FOM, as mentioned above, tfo2 did enhance susceptibility of tfo1 in the double mutant tfo1 tfo2, which was also more susceptible than the tfo1 RFO2/tfo2 heterozygote (Figure 3B). In the Col-0 genetic background, RFO2 expressed resistance in the absence of RFO1 even though resistance conferred by RFO2 showed dependence on RFO1 in the original mapping cross between Col-0 and Ty-0 used to define RFO QTLs (Figure 3C) [9].

In theory, RFO2 might correspond to more than one gene because we discovered RFO2 as a QTL [9]. To address whether At1g17250 alone accounts for the RFO2 QTL, we examined the segregation of resistance in a comparable (tfo2×Ty-0)×Ty-0 mapping population. This new population was similar to our original mapping population with the exception that tfo2 replaced wild type as the Col-0 parent. Specifically, we crossed tfo2 and Ty-0 and then backcrossed the resulting F1 hybrid to Ty-0. As expected, Col-0/Ty-0 heterozygotes and Ty-0/Ty-0 homozygotes appeared in roughly equal proportion with all tested markers (Figure 3C). DNA markers linked to RFO1 and RFO3, which is a third RFO QTL previously detected on chromosome 3 [9], were associated with resistance in both the new and original populations. In contrast, RFO2-C showed significant correlation with
resistance only in the original population (Figure 3C). In the \((\text{g}62\times\text{Ty}-0)\times\text{Ty}-0\) population, resistance at \(RFO2\) instead had a modest correlation with Ty-0 homozygotes (\(RFO2\text{-T/T}\)).

**RFO2 inhibits FOM infection in roots**

Up to now, we equated susceptibility with symptom severity in the above ground foliage, where little if any FOM would be present until late in infection [28]. Possibly, quantitative resistance could reflect reduced symptoms in the expressive phase of infection rather than reduced fungal infection in the below ground roots [32]. To distinguish between these possibilities, we compared the effect of \(RFO1\text{-C}\) and \(RFO2\text{-C}\) on symptoms in shoots and FOM infection in roots. At 12 dpi, \(RFO1\text{-C} RFO2\text{-C}\) exhibited only modest stunting while Ty-0 plants, without the benefit of \(RFO1\text{-C}\) or \(RFO2\text{-C}\), were severely stunted, and older leaves were yellowing (Figure 4A). Meanwhile, \(RFO1\text{-C}\) developed symptoms that were intermediate to those in Ty-0 and \(RFO1\text{-C} RFO2\text{-C}\).

In situ staining with X-Ara reports \(F.\) oxysporum infection as a blue precipitate because \(F.\) oxysporum, and not \(Arabidopsis\), expresses detectable arabinofuranosidase (ABF) activity [28]. Blue staining was stronger and more prevalent in roots of \(RFO1\text{-C}\) than roots of \(RFO1\text{-C} RFO2\text{-C}\) while roots of Ty-0 showed the most extensive staining (Figure 4B); and, uninfected roots of all genotypes remained unstained. The observed differences in X-Ara staining were corroborated by quantifying the accumulation of soluble yellow 4-nitrophenol when roots were incubated with a second substrate of ABF, NP-Ara (Figure 4C).

**RFO2 corresponds to diversity in PSY1R-related RLP genes**

In prior surveys of RLP genes in the Col-0 reference genome, At1g17240 and the neighboring gene \(RFO2/At1g17250\) were identified as a tandem pair of highly-related receptor-like protein (RLP) genes and generically named \(RLP2\) and \(RLP3\), respectively [31,33]. The primary structure of RFO2 is similar to previously characterized RLPs and is comprised of seven domains (Figure S2) [34]: A signal peptide (domains A), four extracellular domains (B through E), a transmembrane domain (F) and a short cytoplasmic tail of nine amino acids (domain G). Most of RFO2 is extracellular and is composed of 23 extracellular leucine-rich repeats (eLRRs, domain C), which are capped at amino-terminal and carboxy-terminal ends by domains B and D, respectively. An acidic domain E joins the extracellular domains to the transmembrane domain. Also, a loop out sequence interrupts the 19th eLRR in domain C.

Genomic sequence in the chromosomal region around \(RFO2\) is highly diverged in Col-0 and Ty-0. In order to characterize the susceptible \(RFO2\text{-T}\) allele, we obtained an 8,311 bp sequence that spans the \(RFO2\) region in Ty-0 using PCR-sequencing. According to BlastN search of the Col-0 reference genome, the best match for the Ty-0 sequence extended across a 12,878 bp interval that included sequence within and between annotated genes.
Figure 3. T-DNA insertion allele rfo2 abolishes RFO2 QTL. (A) Fractions of n F1 hybrids of Ty-0 and Salk_014524 (At1g17200) or Salk_051677 (At1g17250) were susceptible or resistant or had intermediate resistance, according to HI scores at 21 dpi. Median ranks of the two F1 hybrids are dissimilar, according to M-W U test (two-tailed p = 0.021). (B) Fractions of n self progeny of rfo1 RFO2/rfo2, either RFO2/ RFO2, RFO2/rfo2 or rfo2/rfo2, had the lowest, middle or highest third of ranks at 21 dpi. Same italicized letters above genotypes indicates that median ranks were similar, according to M-W U test (two-tailed p = 0.05).

(A) Genotypes of 234 F1 BC progeny of original cross (Col-0 × Ty0) × Ty-0 (top) and 240 F1 BC progeny from new cross (rfo2 × Ty0) × Ty-0 are either Ty-0/Ty0 (T) or Col-0/Ty-0 (C), at RFO1, RFO2- and RFO3-linked markers and marker CHR3.8 that is not linked to a RFO QTL. Fractions of n FOM-infected plants with the lowest, middle or highest third of ranks. Asterisks indicate that alternative genotypes C and T had dissimilar infected plants with the lowest, middle or highest third of ranks. doi:10.1371/journal.pgen.1003525.g003

Arabidopsis lyrata and Arabidopsis thaliana, as depicted in Figure 2. In the shorter Ty-0 sequence, a single RLP gene (RLP2-T) was oriented on the chromosome in the same direction of the in-frame tail of RFO2 and RLP2 in Col-0 (Figure 2). Sequence predicted to be intergenic retained remarkably low nucleotide identity in the two accessions, and intergenic sequence between RFO2 and RLP2 could not be aligned to any Ty-0 sequence. Thus, the Col-0 and Ty-0 variants of RFO2 appeared to be ancestral variation in A. thaliana.

The alignment of coding sequences in the single exons of the three RLP genes at the RFO2 locus showed that RLP2 and RLP2-T were more related to each other than they were to RFO2. Specifically, the 1,956-nucleotide sequence starting at the 5’ end of RLP2-T shared more identity with RLP2 (88 percent) than RFO2 (82 percent). However, a shorter 136 bp sequence at the 3’ end of RLP2-T shared more identity with RFO2 (73 percent) than RLP2 (55 percent). Interestingly, RLP2 shared most identity (92 percent) with the full-length ortholog cRLP2 from Arabidopsis lyrata than even a partially aligned RLP2-T.

From BlastP searches of the Arabidopsis genome database, we learned that RFO2-related RLPs share conspicuous similarity with the extracellular regions of the Arabidopsis RLK PSY1 that perceives the small post-translationally modified tyrosine-sulfated peptide hormone PSY1 involved in cell division and expansion [29]. Specifically, alignment of B and C domains of either RFO2 or RLP2 and PSY1 showed that 74 or 80 percent of residues in the eLRRs, respectively, were identical (Figure S3). Remarkably, RFO2 and RLP2 were more similar to PSY1 than they were to each other as just 73 percent of residues were identical in the alignment of B and C domains of RFO2 and RLP2 (Figure S3). However, outside of the eLRRs, there was little or no sequence conservation between RLK and either RLP, and PSY1 poorly aligned to domains D through G of RFO2 or RLP2 (Figure S4).

The carboxy-end of RFO2 specifies resistance to FOM

By using the same constitutive promoter to express RFO2, RLP2 and RLP2-T, we tested whether differences in transcription could explain why RFO2 conferred resistance while its homologs RLP2 and RLP2-T did not. Coding sequences of the three RLP genes were fused downstream of the constitutive promoter ENTCUP2, and these promoter-gene fusions were introduced to line 1A3 by stable genetic transformation [35,36]. Phosphinothricin (Ppt)-resistant T1 transgenic transformants of line 1A3 exhibited the same wild-type appearance as the untransformed parental line 1A3 (Figure 5A and 5B). Independent T2 lines harboring constitutively-expressed RFO2 (cRFO2) and RLP2 (cRLP2-T) exhibited similar resistance to FOM, in some infection assays, independent T2 lines harboring constitutively-expressed RLP2 (cRLP2) appeared similar to the untransformed parental line 1A3 (Figure 5A and 5B). Meanwhile, a T2 line with constitutive expression of RLP2-T (cRLP2-T) exhibited marginally more resistance than the parental line 1A3, and this resistance was modest in comparison to the resistance conferred by cRFO2 in the same assay (Figure 5C).

Because the resistance of RFO2 was not a consequence of differences in promoter expression of the three RLP genes, we next examined whether resistance could be localized to the
infected roots, in terms of absorbance (OD 410 nm) of 4-nitrophenol.

**Relative Fusarium**

Figure 4. **RFO1-C and RFO2-C restrict infection in roots.** All plants have the genetic background of Ty-0, which lacks both RFO1-C (−) and RFO2-C (−). Line 1A3 and 1A3+tRFO2 also have RFO1-C (+), and only line 1A3+tRFO2 has RFO2-C (+). Plants of each genotype (n = 24) were ranked from most susceptible to most resistant, and plants with median or middle ranks are shown and analyzed. (A) Three representative FOM-infected plants of each genotype are shown at 12 dpi. (B) Roots of FOM-infected plants were stained with X-Ara. (C) Relative Fusarium-derived ABF activity in FOM- (+) and mock- (−) infected roots, in terms of absorbance (OD 410 nm) of 4-nitrophenol formed after 16-hr incubation of whole roots, harvested from (n) mock-infected (−) or FOM-infected (+) plants, with NP-Ara at 10 dpi. Values are adjusted to set mean value of mock-infected Ty-0 roots equal to one. Error bars are confidence interval of the mean (±). Different italicized letters indicate that means are dissimilar, according to Student’s t-test (p < 0.05).

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PSY1R, PSKR1, and PSKR2 promote susceptibility

The sequence conservation of eLRRs in RFO2 and PSY1R prompted us to examine wilt disease progression in loss-of-function mutant psy1 [29]. The function of PSY1R overlaps with the function of two closely related RLK genes PSKR1 and PSKR2 that perceive the tyrosine-sulfated peptide PSK [29,30]. PSK accumulates in cell culture medium and is a key factor permitting the dedifferentiation and redifferentiation of plant cells in culture [37]. Signaling by peptides PSY1 and PSK negatively regulates stress response and senescence, and addition of PSK and PSY1 to agar medium promotes elongation of roots of *Arabidopsis* seedlings [29,30,37]. The functional overlap of PSY1 and PSK signaling prompted us to test the infection of *pskr1* and *pskr2* as well as *psy1r*.

When plants that are insensitive to PSY1 (*psy1r*), insensitive to PSK (double mutant *pskr1 pskr2*) or insensitive to both PSY1 and PSK (*psy1r pskr1 double mutant and *psy1r pskr1 pskr2* triple mutant) were infected with FOM, all mutants were completely resistant. However, the receptor mutants have the Col-0 genetic background, and Col-0 is already completely resistant to FOM. When plants were instead infected with FOC (Figure 6A and 6B) or FOR (Figure 6C), two formae speciales to which Col-0 normally expresses incomplete resistance, mutants were noticeably more resistant than wild type. The triple mutant that is insensitive to both PSK and PSY1 peptides showed the strongest suppression of disease symptoms while mutants that are insensitive to either PSK or PSY1 showed a more modest suppression of disease. X-Ara staining of FOC-infected roots of the triple mutant suggested that initial infection of root tips was normal but indicated that subsequent infection of xylem by *F. oxysporum* was suppressed. We quantified the diminished fungal infection in roots using NP-Ara (Figure 6D), and two-fold less *F. oxysporum*-derived ABF activity was measured in roots of the triple mutant than wild type.

We next examined whether perception of endogenous PSY1 and PSK peptides was critical for the susceptibility that peptide hormone receptor genes expressed in wild type. *Arabidopsis* has a single tyrosyl-protein sulfotransferase gene TPST, and *tpst*
produces only unsulfated and inactive PSY1 and PSK peptides [38,39]. FOC-infected tpt expressed strong resistance that was comparable to the enhanced resistance of psy1r pskr1 pskr2 (Figure 6E), which suggested that endogenous peptide signaling suppressed resistance to *F. oxysporum*.

Resistance of a PSY1R-RFO2 chimeric RLP

Because the eLRRs of RFO2 and PSY1R have remarkable similarity, we examined whether their eLRRs also share a common, interchangeable function by reciprocally swapping their homologous n-domains (Figure S3) and testing the resulting chimeric RLP and RLK genes for function in place of *RFO2* and *PSY1R*, respectively. On the one hand, the chimeric RLP gene *nPcR* was the fusion of sequence coding for the extracellular n-domains of PSY1R (including domains A through C) to sequence coding for the membrane proximal c-domains of *RFO2* (including domains D through G, as depicted in Figure 5A). On the other hand, the chimeric RLK gene *nRcP* was the fusion of sequences coding for n-domains of *RFO2* and c-domains of *PSY1R*.

Even though amino acid similarity in n-domains of RFO2 and PSY1R is comparable to the similarity in functionally equivalent n-domains of *PSY1R* (including domains A through C) to sequence coding for the membrane proximal c-domains of *RFO2* (including domains D through G, as depicted in Figure 5A), the chimeric RLK gene *nPcR* was the fusion of sequences coding for n-domains of *RFO2* and c-domains of *PSY1R*.

Even though amino acid similarity in n-domains of RFO2 and PSY1R is comparable to the similarity in functionally equivalent n-domains of *RFO2*, *RLP2* and *RLP2-T* (Figure S5), n-domains in RFO2 and PSY1R proved to have dissimilar function. For the sake of comparison, we also fused full-length coding sequence of *PSY1R* downstream of the constitutive promoter *ENTCUP2* to make *cPSY1R* (including domains D through G, as depicted in Figure 5A). On the other hand, the chimeric RLK gene *nPcR* was the fusion of sequences coding for n-domains of *RFO2* and c-domains of *PSY1R*.

Unexpectedly, most Ppt-resistant T1 transformants harboring the chimeric RLP gene *nPcR* exhibited obvious pleiotropy. Phenotypes of *nPcR* were reminiscent of the constitutive resistance that is displayed by activated resistance genes in mutants or the autoimmune necrosis, resulting from crosses between particular *Arabidopsis* accessions [40–43]; and, *nPR* transformants of Col-0 or line 1A3 were similarly affected. Indeed, *nPcR*...
of ranks at 18 dpi. Different italicized letters indicates that median ranks of genotypes were dissimilar (M-W U test, \(p<0.05\)). (D) Relative Fusarium-derived ABF activity in FOC- (+) and mock- (−) infected roots, in terms of absorbance (OD410 nm) of 4-nitrophenol formed after 20-hr incubation with NP-Ara, was different at 10 dpi, according to Student’s \(t\)-test (\(n=4\); two-tailed \(p=0.0001\)). Error bars are confidence interval of the mean (\(s=0.05\)). (E) Representative FOC-infected (+) or mock-infected (−) wild type (Col-0) and \(tps\) having median HI scores (\(n=10\) for each genotype) are shown at 18 dpi. doi:10.1371/journal.pgen.1003525.g006

Figure 6. PSY1 and PSK promotes susceptibility to Fusarium wilt. (A) Representative FOC-infected (+) or mock-infected (−) wild type (Col-0) and mutants having median HI scores (\(n=6\) for each genotype) are shown at 24 dpi. (B) Fraction of FOC-infected or (C) FOR-infected plants (\(n=10\) for each genotype) with the lowest, middle, highest third conferred complete resistance to FOC, FOM and FOR as \(nPr\) plants in \(F. oxysporum\)-infected and mock-infected soil were indistinguishable; and, \(X\)-Ara staining detected no vascular infection in \(nPr\) roots at 12 dpi. \(nPr\) transformants had smaller (Figure 7B), often misshapen rose leaves (Figure 7D) that had macroscopic lesions (Figure 7E) and were prone to senescence before wild-type leaves (Figure 7C). \(nPc\) inflorescences were stunted and occasionally arrested by necrosis at their apices. \(nPc\) pleiotropy was dose dependent as phenotypes were consistently less and more severe among \(nPc\) hemizygotes and \(nPc\) homozygotes in the same transgenic line, respectively (Figure 7E); and, phenotypes were more and less severe at high (30°C) and low (22°C) temperatures, respectively. We never observed similar phenotypes in transformants harboring other RLP constructs, including \(RFO2\).

To test whether the c-domains of \(RFO2\) were critical for expression of \(nPc\)-related phenotypes, coding sequences of n-domains of \(PSY1\) and c-domains of RLP2 were fused in the chimeric RLP gene \(nPc2\) (Figure 5A). Ppt-resistant T1 plants harboring \(nPc2\) had wild-type appearance, and T2 plants showed no enhanced resistance to FOM (Figure 5D). Thus, both strong resistance to FOM and visible pleiotropy required the c-domains of \(RFO2\).

Considering that RLPs and RLKs may self-associate as dimers or in oligomeric complexes, we tested whether \(RFO2\) and \(PSY1\) contributed to the pleiotropy of \(nPc\) by examining the effect of \(nPr\) and \(psy1\) on \(nPc\) [44,45]. In a representative \(nPc\) transgenic line 1E9, Ppt-resistance and pleiotropy cosegregated as a single locus. Pure-breeding 1E9 \(nPc\)-resistant F2 of cross \(nPc\)-infected roots, the three possible genotypes of \(RFO2\) segregated with the expected ratio of 1:2:1 (Table 1). When \(F2\) were rank-ordered by size, the median ranks of \(nPc\) and \(nPc\) \(RFO2\) were comparable (two-tailed \(p=0.52\), using Mann-Whitney \(U\) test), and thus \(nPr\) had no effect on the small stature of \(nPc\). Among the \(Ppt\)-resistant \(F2\) of cross \(nPc\times\)\(nPr\), the three possible genotypes of \(RFO2\) segregated with the expected ratio of 1:2:1 (Table 1). When \(F2\) were rank-ordered by size, the median ranks of \(nPc\) and \(nPc\) \(RFO2\) were comparable (two-tailed \(p=0.52\), using Mann-Whitney \(U\) test), and thus \(nPr\) had no effect on the small stature of \(nPc\). Among the \(Ppt\)-resistant \(F2\) of cross \(nPc\×\)\(nPr\), the three possible genotypes of \(RFO2\) segregated with the expected ratio of 1:2:1 (Table 1). However, among the 48 \(Ppt\)-resistant \(nPc\)-infected roots, there were no \(psy1\) homozygotes, and the observed segregation of \(PSY1\) significantly deviated from the expected ratio of 1:2:1 (\(p=0.0003\), Table 1); in fact, numbers of wild-type homozygotes (\(PSY1/PSY1\)) and \(PSY1/psy1\) heterozygotes approximated the ratio (1:2:0) expected for a recessive lethal condition (\(p=0.54\)). To confirm that \(PSY1\) and \(nPc\) were unlinked and \(psy1\) homozygotes were viable in the absence of \(nPc\), we genotyped 32 \(Ppt\)-sensitive \(F2\) and obtained the expected ratio of 1:2:1 for genotypes at \(PSY1\) (Table 1). Thus, viability of \(nPc\)-expressing plants required \(PSY1\).

Because \(PSY1\) is the putative receptor of \(PSY1\), we tested whether the viability of \(nPc\)-expressing plants also required the presence of active \(PSY1\). As \(PSY1\) is unsulfated and inactive in \(tpst\), we crossed \(nPc\) and \(tpst\). In the self \(F2\) progeny of cross \(nPc\×\)\(tpst\), only wild-type homozygotes and \(TPST\)\(tpst\) heterozygotes were identified among 19 herbicide-resistant \(nPc\)-infected \(F2\), and their numbers approximated the 1:2:0 ratio (\(p=0.62\)) and not the 1:2:1 ratio (\(p=0.033\)), whereas \(TPST\) genotypes among 24
RLK genes. With independent T2 lines. Typical phenotypes of representative (indicates that means were similar, according to Student’s confidence interval of the mean (E) macroscopic necrotic lesion at leaf margin. (F) Severity of pleiotropy comparable wild type); (D) malformed, misshapened rosette leaf; and, (left); (C) senescence of leaves (before senescence of leaves of homozygote (right) as compared to leaves of its wild-type Col-0 parent transgenic line 1E9: (B) smaller rosette leaves of four-week-old nPcR

Figure 7. Phenotypes of cRFO2 and cPSY1R chimeric RLP and RLK genes. (A) Mean root length of psy1r, nRcP psy1r and cPSY1R psy1r after two weeks of growth on vertical PN agar plates. Error bars are the confidence interval of the mean (α = 0.05). Same italicized letter indicates that means were similar, according to Student’s t test (p > 0.05; for all genotypes, n = 15). Similar results were reproduced with independent T2 lines. Typical phenotypes of representative nPcR transgenic line 1E9: (B) smaller rosette leaves of four-week-old nPcR homozygote (right) as compared to leaves of its wild-type Col-0 parent (left); (C) senescence of leaves (before senescence of leaves of comparable wild type); (D) malformed, misshapened rosette leaf and, (E) macroscopic necrotic lesion at leaf margin. (F) Severity of pleiotropy cosegregated with herbicide resistance marker linked to nPcR in self progeny of 1E9 hemizygote: Progeny of plants with normal appearance (wild type, Wt) were all Ppt-sensitive (−/+), progeny of the most affected were all Ppt-resistant (+/+), and Ppt resistance and sensitivity segregated (+/−) in progeny of plants with intermediate phenotypes. doi:10.1371/journal.pgen.1003525.g007

herbicide-sensitive F2 approximated the 1:2:1 ratio (p = 0.88, Table 1). Thus, nRcR-expressing plants depended on the presence of active, sulfated peptides, including PSY1, and the presence of PSY1R. Discussion

Phylogenic analysis implicates most of the 90 and 57 RLP gene sequences in the reference genomes of rice and Arabidopsis, respectively, in host response to biotic stress [34]. Most RLP genes are members of species-specific clades and (76 percent in rice and 58 percent in Arabidopsis) are clustered at loci with two or more related genes [33]. Species-specific genes and gene clustering are also features of the NB-LRR family of resistance genes and imply that lineages of RLP genes are expanding, contracting and diversifying to meet the evolving challenge of infectious disease [46,47]. Indeed, reverse genetic approaches show that loss-of-function mutations in three Arabidopsis RLP genes quantitatively compromise innate immunity to virulent and nonhost pathogens [31,48,49].

However, diversity in RLP genes has not been associated with a disease resistance trait in Arabidopsis or rice until now. In cultivated species such as tomato, RLP resistance traits are typically the result of interspecific breeding or the inadvertent propagation of loss-of-function polymorphisms [50,51]. The highly diverged RFO2 alleles suggest that diversity in RLP genes contributes to quantitative variation in resistance in wild species.

Although monogenic resistance traits are usually associated with NB-LRR genes, several RLP genes confer strong monogenic resistance to specific pathogens as well [52]. The strong pathogen-specific resistance of transgenic RFO2 is reminiscent of such gene-for-gene resistance. The best-studied RLP genes are in the Cf clade and mediate resistance to specific races of the foliar fungal pathogen Cladosporium fulvum that express corresponding avirulence genes [50]. Meanwhile, apple Vfa1 and Vfa2 confer resistance to five of the obligate fungal pathogen Venturia inaequalis, tomato LeEix2 confers recognition of an ethylene-inducing xylanase from biocontrol fungus Trichoderma viride, oilseed rape Leptosphaeria maculans expressing AvrLm1, and tomato Vel confers strong resistance to races of Verticillium species expressing avirulence gene Ave1 [53–56]. Interestingly, Vel also confers modest quantitative resistance to virulent F. oxysporum forma specialis lycopersici [33]. Likewise, we presume that RFO2 perceived an extracellular Fusarium-derived signal that was present in FOM infection and absent in FOC infection [57]. However, we cannot discount that FOC infection suppressed RFO2’s perception of a signal that was present in all F. oxysporum infections. In either case, once induced, RFO2 was effective against all three crucifer-infesting formae speciales as the constitutive resistance of nPcR lacked specificity.

PSY1 and PSK signaling compromised resistance to vascular infection by F. oxysporum. Recently, Igarashi et al. reported that psy1l (but not psk2l) is more resistant to leaf infection by virulent P. syringae [58], PSY1R, PSKR1 and PSKR2 were identified and characterized for perception of PSY1 and PSK and for the effects that this perception has on root growth, cell proliferation and senescence [29,30]. Igarashi et al. suggest that PSK signaling directs allocation of resources between energy-intensive processes,
toward growth and away from immunity [58]. However, PSK and PSY1 signaling more fully influences the longevity and growth potential of mature differentiated cells [29], and absence of peptide signaling in the triple mutant arguably had a more modest effect on plant mass than wilt resistance (in Figure 6A). Natural resistance traits, such as RFO1, RFO2 and tomato Immunity genes, promote resistance to Fusarium wilt by inhibiting infection in the vascular cylinder [2,28]. Because PSK depresses stress responses in general and immunity in particular, we suspect that peptide signaling depressed the considerable but incomplete resistance of Col-0 to FOC and FOR [9,37,58,59]. The strong resistance of tpst suggests that endogenous PSY1 and PSK depressed immunity, though the expression of other proteins with tyrosine sulfation, including root meristem growth factors, is also affected by tpst [60]. The strong wilt resistance of the receptor triple mutant does not tell us whether FOC or FOR normally exploits PSY1 and/or PSK signaling to induce susceptibility; however, it does demonstrate that manipulation of even basal signaling would be a fruitful target for pathogen effectors.

Amino acid identity in the eLRRs of RFO2 and PSY1R is conspicuous because RLPs and RLKs usually lack meaningful sequence conservation beyond the structural constraints of the eLRR motif [33]. Premature termination of translation in an RLK gene, such as Xa21D, may give rise to a residual RLP-like gene [61]. However, RFO2 is not simply a truncation of PSY1R as the RFO2-related RLPs and PSY1R have little if any sequence conservation outside of the eLRRs (see Figure S4). The regular presence of PSY1R-related RLK genes and sporadic distribution of RFO2-like RLP genes in plant genomes in the Phytozome v8.0 database presumably reflects the distinct roles of these genes in peptide signaling and defense response, respectively (A.D., unpublished data) [62].

In spite of the relatedness of eLRRs of RFO2 and PSY1R, we failed to connect RFO2 to a role in PSY1 signaling in normal root growth. PSY1 supplementation enhances root growth, and roots of psy1r are shorter than wild-type (Figure 7A) [29]. However, neither transgenic expression of RFO2 nor deficiencies (pd2) of RFO2 affected root length; and, we found that root lengths of psy1r and psy1r tpst were comparable (Y.S., unpublished data).

RFO2’s similarity to PSY1R and lack of function in PSY1 signaling are consistent with the decoy model for perception of pathogen effectors [63]. In theory, effectors that target PSY1R might select for a decoy receptor, such as RFO2, that mimics the interaction between effectors and PSY1R but lacks the function that effectors are targeting. Because PSY1 signaling suppressed immunity to F. oxysporum infection, the relevant effector would be an agonist or positive regulator of PSY1R. Although how PSY1R perceives PSY1 is unknown, PSK directly bind to the PSK receptor, and a photo-activated analog of PSK preferentially labels the loop out sequence within eLRRs [64].

| Parental cross | nPcRb | Geneb | Nc | Genotype of F2 | pbd |
|---------------|-------|-------|----|---------------|-----|
| nPcR x rf62 | + | rf62 | 51 | H | 0.69 |
| nPcR x pskr2 psy1 | + | pskr2 | 45 | W | 0.91 |
| nPcR x tpst | + | tpst | 19 | M | 0.0003 |
| nPcR x tir3 | + | tir3 | 32 | H | 0.033 |

*F2 progeny were selected for Ppt resistance (+), which cosegregates with nPcR or sensitivity (−).

1See Materials and Methods for codominant DNA markers used to genotype particular genes.

2Number of F2 plants genotyped.

3Probability from chi-squared test that observed genotypes fit expected 1:2:1 ratio, derived from random segregation of wild-type and mutant alleles. Degrees freedom = 2. p values less than 0.05 are in bold type.

4Observed genotypes were wild-type homozygotes (W), mutant homozygotes (M) and heterozygotes (H).

5Expected monohybrid segregation ratio is 1 wild type : 2 heterozygotes : 1 mutant.

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PFO2 Implicates PSY1 Signaling in Resistance

Table 1. F2 segregation of mutants in crosses with transgene nPcR.

In spite of the relatedness of eLRRs of RFO2 and PSY1R, we failed to connect RFO2 to a role in PSY1 signaling in normal root growth. PSY1 supplementation enhances root growth, and roots of psy1r are shorter than wild-type (Figure 7A) [29]. However, neither transgenic expression of RFO2 nor deficiencies (pd2) of RFO2 affected root length; and, we found that root lengths of psy1r and psy1r tpst were comparable (Y.S., unpublished data).

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The critical role of the c-domains of RFO2 in both the FOM-specific resistance of cRFO2 and constitutive resistance of nPcR suggests that the pleiotropy of nPcR is the aberrant, constitutive activation of resistance that FOM normally induces via RFO2. When the c-domains in RL2, which expressed no resistance to FOM, replaced c-domains of RFO2 in cRFO2 and nPcR, the resulting RLs nR2 and nPcR expressed neither resistance to FOM nor visible pleiotropy. If pleiotropy were simply the consequence of expressing a truncated PSY1R without a kinase domain, nPcR would also express visible pleiotropy.

In an attempt to explain the constitutive resistance of nPcR, we recalled that PSY1R perceived endogenous PSY1 while RFO2 appeared insensitive. When the n-domains of RFO2 and PSY1R were swapped, the n-domains of RFO2 appeared insensitive to PSY1 in the chimERIC RLK nR2P, which failed to suppress the reduced root growth of psl1r (Figure 7A). We reasoned that RFO2 was only activated by an FOM-derived signal, while nPcR was continuously activated by endogenous PSY1. If this hypothesis were correct, simply removing endogenous PSY1 should abolish the pleiotropy of nPcR.

Contrary to expectation, absence of active sulfated peptides, including PSY1, in psl1r as well as loss of the PSY1 receptor in psl1r exacerbated the phenotype of nPcR. We imagine that plants with the psl1r nPcR and psl1r nPcR genotypes were not recovered because constitutive resistance had attained a lethal level of expression. This would be consistent with the obvious effect that a two-fold difference in nPcR copy number in hemizygotes and transgene homozygotes had on nPcR phenotypes (Figure 7F). [66,67]. Genetic analysis discounted trivial explanations for why psl1r nPcR and psl1r nPcR were not recovered, such as linkage between the nPcR transgene and mutation. Thus, paradoxically, we found that PSY1 was not required to activate resistance via nPcR, rather PSY1 signaling was negatively regulating the constitutive resistance of nPcR.

We considered the possibility that PSY1 and PSK signaling indirectly suppressed the constitutive resistance of nPcR in wild type. Because psl1r pslk1 pskr1 and psl1 strongly enhanced resistance to F. oxysporum infection, it was possible that an enhanced defense response in psl1t permitted the constitutive resistance of nPcR lethal. On the other hand, psl1r had a much more modest effect on resistance to F. oxysporum than the triple receptor mutant or psl1t, so it seemed remarkable that psl1r would have as profound an effect on viability as psl1t. Nevertheless, if basal signaling of PSY1 and PSK were suppressing the constitutive resistance of nPcR, we reasoned that inducing PSK signaling by supplementation with PSK should counteract constitutive resistance and improve growth of nPcR seedlings, including roots. According to Igarashi et al., exogenous PSK can suppress elicitor-induced root growth inhibition [38]. However, we found that added PSK failed to have an appreciable effect on the abbreviated root growth of nPcR seedlings or improve the appearance of nPcR seedlings, even as PSK was able to stimulate the root growth of wild-type and psl1r seedlings (Figure S6). Furthermore, pslk2 nPcR, which has a partial deficiency in PSK signaling, was recovered from crosses and was indistinguishable from nPcR siblings, though both pslk2 and psl1r contributed to resistance to F. oxysporum (in Figure 6). Because manipulation of PSK signaling failed to alleviate or exacerbate the pleiotropy of nPcR, PSY1 signaling appeared to be intimately associated with negative regulation of nPcR.

To account for the PSY1-dependent negative regulation of nPcR, we hypothesized that an activity-dependent negative feedback mechanism that normally controls the expression of PS1R1 also controls the expression of nPcR (Figure 8A). In this scenario, engagement of PSY1 and PSY1R has two consequences, (i) transduction of PSY1 signaling and (ii) downregulation of PSY1-activated PSY1R. Engagement of PSY1 and nPcR, on the other hand, targets nPcR for downregulation but does not transduce the PSY1 signal. Interestingly, endocytosis is proposed to have a prominent role in attenuating PAMP signaling [68]. Downregulation of the flagellin receptor FLS2 by its synthetic peptide ligand flg22 is a clear precedent for negative feedback in RLK signaling in plants [69]. Engagement of FLS2 and flg22 recruits the coreceptor BAK1, which concomitantly promotes FLS2 signaling as well as proteosome-mediated degradation of FLS2 [70].

Importantly, the apparent negative regulation of nPcR by PSY1 signaling suggests a connection between PSY1 signaling and RFO2-mediated resistance. The mechanism that normally downregulates PSY1-activated nPcR presumably targets a common structural feature in PSY1R and nPcR, which is the extracellular n-domains of PSY1R. Conservation of the corresponding n-domains of RFO2 and PSY1R implies that RFO2 is also a target of the same negative regulation.

Because constitutive resistance of nPcR was not dependent on PSY1 [in psl1t nPcR], we needed an alternative explanation for the different phenotypes of cRFO2 and nPcR. Both cRFO2 and nPcR encode the same c-domains of RFO2, so it must be the n-domains of nPcR that constitutively provide resistance and the n-domains of RFO2 that constitutively provide no resistance [in the absence of FOM infection]. Considering this, we hypothesize that the cLRRs of RFO2, acting as a decoy, mimic a state of the cLRRs of PS1R that is already PSY1-activated and competent for downregulation. Intrinsically-activated cLRRs in RFO2 would account for their insensitivity to PSY1. Being competent for downregulation, RFO2 would be constitutively downregulated, even if no PSY1 signaling were present, and thus would normally express no resistance. On the other hand, nPcR, which needs activation by PSY1 in order to be downregulated, would constitutively express resistance in those tissues where, and at times when, there is insufficient PSY1 to fully downregulate nPcR (Figure 8B).

We present a model to explain how an effector could induce PSY1 signaling without directly engaging PSY1R, and how RFO2 could directly or indirectly perceive such an effector. By expressing an effector that inhibits the PSY1-dependent negative feedback mechanism, FOM could stabilize PSY1-activated PSY1R (Figure 8C). Just as psl1r and psl1t were able to upregulated the expression of resistance by nPcR by abolishing PSY1-dependent downregulation, an effector could upregulate PSY1 signaling by inhibiting the downregulation of PSY1-activated PSY1R and thereby suppress host immunity. However, in plants expressing RFO2, the effector would likewise stabilize RFO2, acting as a decoy for the downregulation-competent PSY1R. Stabilized and upregulated RFO2 would induce robust defense response (Figure 8D).

There are two appealing aspects to this model. For one, existence of negative feedback in PSY1 signaling explains why a pathogen targets this mechanism rather than secretes a PSY1-like ligand. Chronic PSY1 signaling would be achieved more effectively by stabilizing endogenous PSY1-activated PSY1R if perception of excess PSY1-like ligand were suppressed by downregulation of the receptor. For two, RFO2 behaves as a guard protein and does not need to directly engage the effector that it recognizes. If an effector were to inhibit any component of the negative feedback mechanism, RFO2 would be activated. Thus, RFO2 functions as a guard protein for the negative feedback mechanism.

Simply changing the transcriptional context or copy number of RFO2 in transgenic plants converted a modest quantitative resistance trait into a strong resistance gene. No aberrant visible
phenotype accompanied the stronger resistance of transgenic RFO2, and resistance remained specific to FOM. We wonder whether gene expression rather than protein structure limits the strength of other resistance QTLs as well. Effectiveness of some qualitative gene-for-gene resistance traits is restricted, for instance, to a developmental stage, which suggests a partial deficiency in gene expression [71,72]. Some opinion holds that resistance QTLs are in fact weak gene-for-gene resistance traits [21,26]. If our experience of limited gene expression were commonplace, the potential utility of genes underlying resistance QTLs might be underappreciated.

Finally, our initial analysis of RFO2 has produced a testable model to account for RFO2-mediated resistance. Future work should establish the biochemical nature of PSY1-dependent negative regulation of nPcR. In addition, identification of the relevant F. oxysporum PAMP(s) and/or effector(s) should prove especially useful for molecular characterization of this resistance mechanism.

Materials and Methods

Arabidopsis and plant growth conditions

Salk insertion lines and BAC DNA clones, F6I1, F20D23 and F28G4 were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH). Seeds of psy1r, psy1r pskr1, pskr1 pskr2, psy1r pskr1 pskr2 and yst were provided by Dr. Y. Matsubayashi (Nagoya University, Nagoya, Japan). Ty-0, rfo1 and lines 1A3 and 4D2 were derived from F1BC plants in [9]. Plants were grown on Jiffy7 peat pellets (Growers Solution, Cookeville, TN) under cool white fluorescent lighting with moderate intensity with 12-hr daylength and 28°C daytime and 26°C nighttime temperatures. Seedlings were grown from bleach-sterilized seeds on Petri plates with plant nutrient (PN) minimal medium and 0.8% agar alone or, for antibiotic selection, with 0.5% sucrose [73]. Transgenic seeds were selected with kanamycin (50 mg/L) or phosphinothricin (Ppt, 20 mg/L). Phytosulfokine-α was purchased from PolyPeptide Laboratories, Inc., Torrance, CA. The Arabidopsis Information Resource (TAIR, www.arabidopsis.org) provided reference genome sequence and annotation.

Infections with F. oxysporum

FOC, FOR and FOM originate from P.H. Williams through H.C. Kistler [11,74]. F. oxysporum cultures were grown on Czapek-Dox minimal medium (Oxoid Ltd., Hampshire, UK), and conidia were harvested from 3- to 5-d shaken cultures, washed 3 times and resuspended in sterile water. For infection, conidial density was adjusted to between 10^6 to 10^8 conidia/mL, using a hemacytometer, and 2- to 3-wk old plants were irrigated with conidial suspension or water (for mock infection). Disease symptoms were scored between 12 and 24 days post infection (dpi) using a health index (HI), previously called a disease index in [9], with ordinal ratings of one (dead) to five (unaffected) in steps of 0.5. Plants that were deemed susceptible typically had HI > 3, or resistant had HI ≤ 4 or were scored as having intermediate resistance if...
3≤HI<4. For statistical analysis, plants were rank-ordered, from most susceptible to most resistance, and Mann-Whitney U test was used to evaluate the ranks of different genotypes. Sometimes rank-order was derived from multiple HI scores, recorded at two or more time points, in which case later HI scores had priority over earlier scores. From rank-order, plants with the lowest, highest or middle third of ranks were arbitrarily deemed susceptible, resistant or had intermediate resistance, respectively.

Mapping RFO2

Linkage analysis of 80 FOM-infected progeny from the cross of 1A3 and 4D2 mapped RFO2 between flanking SSLP markers CIW12 and F21M12 [9]. To confirm RFO2 genotypes of plants with informative recombination breakpoints, we tested the cosegregation of Rfo2 phenotype (resistance to FOM) and genotype of an RFO2-linked marker in 25 to 50 progeny. If Rfo2 phenotype and marker genotype cosegregated, the genotype was RFO2-C/T; and, if Rfo2 phenotype and marker genotype assorted independently, the genotype was RFO2-T/T or RFO2-C/C. A fine-map position for RFO2 was obtained by screening for recombination breakpoints in the CIW12-F21M12 interval among 200 uninfected progeny from cross (1A3×4D2) and the 248 original F1/BC progeny (see Table S1 for description of SSLP markers for fine-mapping) [9]. In particular, Rfo2 phenotype co-segregated with RFO2-linked markers in progeny of 4E3 and 1B9 that have breakpoints on either side of RFO2 (see Figure S5A). The interval between breakpoints in 4E3 and 1B9 was less than the 258 kb between SSLPs F11A6 and F17F16 (see Figure S3B).

Cloning RFO2

In total, 25 Col-0 genomic restriction fragments of 3 BAC clones F611, F28G4 and F20D23, representing 50 of 68 genes in the RFO2 interval (see Figure S1), were subcloned into binary vector pPZP212 [73] for Agrobacterium tumefaciens-mediated transformation of line 1A3. Kanamycin resistance selected for stable integration of Col-0 genomic subclones. Relative HI scores of multiple FOM-infected T1 and/or T2 transplants as well as untransformed 1A3 were used to assign Rfo2 phenotypes to subclones. A summary of all Col-0 genomic subclones and their Rfo2 phenotypes is in Figure S1.

Sequencing RFO2 in Ty-0

RFO2-T sequence (Genbank accession HQ141412) was a contig assembled from PCR-sequencing. Both strands of four overlapping PCR products amplified from Ty-0 DNA were sequenced. Primer sequences, sizes of PCR products and lengths of sequence overlap are in Table S2. Best-matched sequence to RFO2-T in TAIR10 reference genome database was between nucleotides 5,893,811 to 5,906,689 on chromosome 1, according to BlastN 2.2.8 search function at TAIR. DNA similarity search tool YASS (http://bioinfo.lifl.fr/yass/) assisted the hand-edited alignment of intergenic regions in Col-0 and Ty-0 sequences [76]. The percent nucleotide identity was calculated in a 75-nucleotide window centered at a nucleotide position, and mismatched nucleotides and gaps of any length in the alignment of Col-0 and Ty-0 sequences were discounted equally. Sequence of the Arabidopsis lyrata ortholog AIRLP2 (gene 920636) was from the Phytozome v8.0 plant genome database.

Genotyping

DNA markers were PCR-amplified from crude leaf preparations and analyzed as in [77]. PCR primers for genotyping pso1, rfo2, psk2 and psy1r are in Table S3.

Genotypic and phenotypic analysis of rfo2

At least five plants for each of 30 homozygous Salk T-DNA lines (listed in Table S4) were infected with 5×10^7 FOM conidia/mL. Four lines (Salk_014324, Salk_051677, Salk_049366 and Salk_095714) were crossed to Ty-0, and resulting F1 were infected with 10^7 FOM conidia/mL. F1 progeny of (Psy2×Ty-0)×Ty-0 as well as the original (Col-0×Ty-0)×Ty-0 population [9] were genotyped with RFO1-, RFO2-, RFO3-linked and RFO-unlinked Col-0-specific dominant markers (Table S5). Dominant marker primers were used with the Qiagen Multiplex PCR kit (Qiagen Inc., Valencia, CA). FOM-infected F1/BC populations were rank-ordered using HI scores recorded at 12, 15 and 18 dpi. Lowest, middle and highest third of ranks were designated susceptible, intermediate resistance and resistant, respectively.

Chimeric RLP and RLP transgenes

BanHH and SpeI, or SpaI and NepI sites were introduced to 5’ and 3’ ends of PCR-amplified sequence coding for n- or c-domains, respectively, using PCR primers with restriction sites at 5’ ends (Table S6). Sequences coding for n- and c-domains of RFO2, RLP2 and PSY1R or RLP2-T were PCR-amplified from Col-0 or Ty-0 DNA. DNA sequencing was used to verify the sequence of PCR-amplified subclones. Restriction fragments coding for n- and c-domains were ligated to BanHH- and NdeI-digested binary vector pORE-E3 to make cRFO2, cRLP2, cRLP2-T and cPSY1R expression constructs [36]. To make chimeric constructs, BanHH- and SpeI-digested DNA for n-domains in cRFO2, cRLP2, cRLP2-T and cPSY1R expression constructs were exchanged using DNA ligation. In pORE binary vectors, gene constructs were located in T-DNA and were ready for transfer to plants using A. tumefaciens GV3101 [36,78]. Resistance to Ppt selected for seedlings with stably integrated constructs, and the presence of chimeric gene sequences in transformed Ppt-resistant plants was verified by PCR.

Visualizing and quantifying glycosidase activity in roots

Cleaning and staining of roots with 5-bromo-4-chloro-3-indolyl-α-L-arabinofuranoside (X-Ara), 4-nitrophenyl-α-L-arabinofuranoside (NP-Ara), purchased from Gold Biotechnologies Inc. (St. Louis, MO) is described in [28]. To quantify Fusarium-derived arabinofuranosidase activity, freshly harvested roots were incubated with 0.04% NP-Ara for 16 h at 28°C in 30-fold excess staining solution.

Phylogenic analysis

Coding sequences and translated sequences of RFO2, RLP2, RLP2-T, PSY1R (Atg1g72300) and PSK2 (Atg2g02220) in the TAIR10 genome and proteome databases were aligned using the Clustal method and default settings in MEGA5 [79].

Supporting Information

Figure S1 Resistance phenotype of Col-0 subclones in the RFO2 interval. The RFO2 interval (258 kbp between nucleotides 5,766,000 and 6,023,500 in TAIR10 reference genome sequence for chromosome 1) was defined by a recombinant breakpoint in lines 4E3 between SSLPs F11A6 (at nucleotide 5,766,000) and F20D23 (at nucleotide 5,820,000), on the low end, and a recombination breakpoint in line 1B9 between SSLPs F28G4 (at nucleotide 5,943,000) and F17F16 (at nucleotide 6,023,500), on the high end. Resistance phenotypes (Rfo) of Col-0 genomic clones were tested in T1 and/or T2 transformants of line 1A3. Horizontal bars are proportional to the sequence length of subcloned Col-0 DNA and extend across their respective positions in the genomic interval.
below. Bars are labeled with the subclone names and, in parentheses, the sizes and gene content of subcloned sequence (to the right). Fifty of the 68 genes in the RFO2 interval were included in at least one construct; Xba1.5 includes nucleotides 5777507 to 5790031 and genes AT1G16900, AT1G16905, AT1G16910, AT1G16916 and AT1G16920; Xba1.4 includes nucleotides 5791422 to 5801901 and genes AT1G16940, AT1G16950 and AT1G16960; Pac1.4 includes nucleotides 5810084 to 5824597 and genes AT1G17000, AT1G17010, AT1G17020 and AT1G17030; Pac1.3 includes nucleotides 5824895 to 5833233 and genes AT1G17040 and AT1G17050; Kpn1.4 includes nucleotides 5833071 to 5841463 and genes AT1G17060, AT1G17070 and AT1G17080; Xba1.3 includes nucleotides 5837555 to 5843587 and genes AT1G17070, AT1G17080 and AT1G17090; Kpn1.3 includes nucleotides 5846018 to 5854394 and genes AT1G17110 and AT1G17120; Pac1.2 includes nucleotides 5850466 to 5860625 and genes AT1G17120, AT1G17130, AT1G17140, AT1G17145 and AT1G17147; Pac1.1 includes nucleotides 5866025 to 5878981 and genes AT1G17150, AT1G17160, AT1G17170, AT1G17180 and AT1G17190; Sal1.4 includes nucleotides 5867417 to 5882030 and genes AT1G17160, AT1G17170, AT1G17180, AT1G17190 and AT1G17200; Sal1.3 includes nucleotides 5882030 to 5891121 and genes AT1G17210 and AT1G17220; Xba1.1 includes nucleotides 5897230 to 5981727 and genes AT1G17200, AT1G17210 and AT1G17220; Kpn1.2 includes nucleotides 5898670 to 5904885 and genes AT1G17220, AT1G17232, AT1G17230, AT1G17235, AT1G2740 and AT1G2750; Sal1.2 includes nucleotides 591121 to 5914913 and genes AT1G2732, AT1G2730, AT1G2735, AT1G2740, AT1G2750, AT1G2760, AT1G2770 and AT1G2775; Kpn1.1 includes nucleotides 5905771 to 5921349 and genes AT1G2760, AT1G2770, AT1G2775, AT1G2777, AT1G2780 and AT1G2785; Sal1.1 includes nucleotides 59323219 to 5946989 and genes AT1G27340, AT1G27345 and AT1G27350; Xma1.1 includes nucleotides 5970942 to 5986501 and genes AT1G27420, AT1G27430 and AT1G27440; Avr2.1 includes nucleotides 6007806 to 6004198 and genes no full-length cDNA; Nsi1.3 includes nucleotides 6003646 to 6008883 and genes AT1G2732, AT1G2730, AT1G2735 and AT1G2740; BamH1.1 includes nucleotides 5900529 to 5901022 and genes AT1G2732, AT1G2730, AT1G2735 and AT1G2740; AgeI.1 includes nucleotides 5902722 to 5903905 and genes AT1G2732, AT1G2730, AT1G2735, AT1G2740 and AT1G2750; Hind3.1 includes nucleotides 5904051 to 5905229 and genes AT1G2732, AT1G2725, AT1G2740 and AT1G2750; Nsi1.1 includes nucleotides 5907957 to 5908644 and genes AT1G2750 and AT1G2760; and, Nsi1.2 includes nucleotides 5904083 to 5906444 and gene AT1G17250.  

Figure S2 Domain structure of RFO2. The amino acid sequence of RFO2, in single-letter code, is divided into seven alphabetically named domains. Sequence in the C domain is highlighted in bold [34]. In domain E, acidic residues are highlighted in bold. In domain F, a predicted transmembrane sequence is underlined, and a conserved GxxxG motif is highlighted in bold [33].  

Figure S3 Alignment of eLRRs (domain C) of PSY1R-like proteins and PSKR1. Alignment of the translated amino acid sequences of PSY1R, RFO2, RLP2 (RLP2c), RLP2-T (RLP2t) and PSKR1, encoding domain C, are shown in single-letter code. All residues that are identical to PSY1R are highlighted by white type on black background. The amino acid position from the start codon is given for the leftmost residue in each line.  

Figure S4 Alignment of PSY1R and RFO2-like RLP sequences at the carboxy-terminal ends of RLPs. Translated sequences of RFO2, RLP2 (RLP2c) and RLP2-T (RLP2t), encoding carboxy-terminal ends of RLPs, are aligned to the translated sequence of PSY1R in single-letter code. Amino acid residues that are identical in >50 percent of sequences are highlighted by white type on black background. The amino acid position from the start codon is given for the leftmost residue, and asterisks are stop codons. A SDel restriction site (5'-ACT-AGT-3'), which codes for the threonine (T) and serine (S) residues at the arrow, was introduced as a silent mutation into coding sequences for the creation of chimeric fusions among RLP and RLK genes.  

Figure S5 Recombination breakpoints defining RFO2 map position. (A) Fractions of n F2 from cross 1A3×4D2 that were susceptible (HI scores <2, open column), had intermediate resistance (2≤HI scores <4, half-filled) or were resistant (HI scores ≥4, filled) at 18 dpi. Only F2 of F1 plants 4E3, 1B9 and 5E1 from cross 1A3×4D2 that were either homozygous Ty-0 (T/T) or Col-0/Ty-0 heterozygotes (C/T) at RFO2-linked markers as well as C/T at RFO1-linked marker F19K16 are shown. (B) Genotypes, either Ty-0 (T) or Col-0 (C), at RFO2-linked markers (above) on the single recombination chromosomes in F1 plants 4E3, 5E1 and 1B9 from cross 1A3×4D2. Marker intervals with a crossover are marked with ‘X’. Number of TAIR10 annotated open reading frames (ORFs) in marker intervals is given below. Rfo2 phenotype of F1 plants (on the right) was evaluated in F2 progeny (in A).  

Figure S6 Root growth of npr1 is unaffected by PSK peptide. Two-week-old seedlings of Col-0 (wild type), psy1r, psy1r psy1r psy2r and npr1 (line 1E9) were grown from seeds sown on vertically-oriented PN agar plates with (+) or without (−) added PSK (0.1 μM). (A) Lengths of PSK-treated wild-type and psy1r roots (n = 20) were longer than untreated roots (n = 20), according to Student’s t test (two-tailed p = 0.044 and 0.015, respectively) while length of PSK-treated and untreated psy1r roots (n = 20) had similar lengths (p = 0.60). (B) Length of PSK-treated wild-type roots (n = 20) were longer than untreated roots (n = 20), according to Student’s t test (two-tailed p = 0.005) while length of PSK-treated and untreated npr1 roots (n = 20) had similar lengths (p = 0.16).  

Table S1 PCR primers for mapping and genotyping RFO2 and RFO1.  

Table S2 PCR primers and products for sequencing RFO2 in Ty-0.
Table S3  PCR primers for genotyping Salk insertions.  
(PDF)

Table S4 Homozygous T-DNA insertion lines in RFO2 genetic interval.  
(PDF)

Table S5 PCR primers for dominant multiplex PCR markers.  
(PDF)

Table S6 PCR primers for constructing RLP and RLK genes.  
(PDF)

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